

# **ROLE OF THE VAGUS NERVE AND INTRINSIC CARDIAC GANGLIA IN BOTH REMOTE AND LOCAL MYOCARDIAL ISCHAEMIC CONDITIONING**

Thesis submitted by

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## **DECLARATION**

I, Jack Michael Jarman Pickard, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis. All technical assistance relevant to the results presented herein is duly acknowledged.



*This thesis is dedicated to  
my parents, Ed and Maria*

# **ABSTRACT**

## **Background**

There is a clear clinical need for interventions that limit the extent of injury associated with myocardial ischaemia-reperfusion injury (IRI). Two such interventions are classical (IPC) and remote (RIC) ischaemic preconditioning. The mechanism of action in both cases involves release of one or more humoral factors. However, the existence of a neural pathway remains equivocal.

The aim of this thesis was to investigate the importance of a neural pathway in both classical and remote ischaemic conditioning, with particular focus on the vagus nerve and intrinsic cardiac ganglia. In addition, the efficacy of non-invasive vagus nerve stimulation was investigated as a novel cardioprotective intervention. Finally, we performed a meta-analysis investigating the efficacy of RIC in animal *in vivo* models of myocardial infarction.

## **Methods and Results**

This thesis first aimed to investigate the co-dependence between the neural and humoral pathways of RIC. Bilateral cervical vagotomy abolished release of the humoral blood-borne mediator. Moreover, pharmacological antagonism of intrinsic cardiac ganglia abrogated RIC-mediated protection.

This thesis was the first to reveal a neural component to the mechanism of IPC. We suggest a sensory feedback loop in response to IPC, involving activation intrinsic cardiac ganglia and post-ganglionic parasympathetic fibres projecting to the ventricles.

Finally, transcutaneous vagus nerve stimulation (tVNS) in healthy human volunteers induced release of a blood-borne cardioprotective factor. However, tVNS was not significantly cardioprotective in an *in vivo* rat model of IRI.

## **Conclusions**

Release of the humoral blood-borne mediator following RIC is dependent on prior vagus nerve activation. Furthermore, intrinsic cardiac ganglia are recruited as part of the mechanism of both RIC and IPC. Finally, tVNS is suggested to be a novel cardioprotective intervention.

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## PUBLICATIONS

Below details publications arising from the work presented in this thesis

### Original research articles:

**Pickard JMJ, Burke N, Davidson SM, Yellon DM (2017)** *Intrinsic cardiac ganglia and acetylcholine are important in the mechanism of ischaemic preconditioning. Basic Res Cardiol 112:11. doi: 10.1007/s00395-017-0601-x*

**Pickard JMJ, Davidson SM, Hausenloy DJ, Yellon DM (2016)** *Co-dependence of the neural and humoral pathways in the mechanism of remote ischaemic conditioning. Basic Res Cardiol 111:50. doi: 10.1007/s00395-016-0568-z*

**Bromage DI \*, Pickard JMJ \*, Rossello X, Ziff OJ, Burke N, Yellon DM, Davidson SM (2016)** *Remote ischaemic conditioning reduces infarct size in animal in vivo models of ischaemia-reperfusion injury: a systematic review and meta-analysis. Cardiovascular Research. doi: 10.1093/cvr/cvw219*

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### Review articles:

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**Sivaraman V, Pickard JMJ, Hausenloy DJ (2015)** Remote ischaemic conditioning: cardiac protection from afar. *Anaesthesia* 70:732–48. doi: 10.1111/anae.12973

**Abstract articles:**

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\* = authors contributed equally to this work

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## **Chapter 1 GENERAL INTRODUCTION**

### **1.1 Coronary artery disease**

Coronary Artery Disease describes the group of conditions that precipitate from the formation of an atherosclerotic plaque in one or more coronary arteries. Progressive accumulation of lipids and fibrous elements in the artery wall results in a protrusion of the plaque into the lumen, thereby reducing blood flow to the downstream myocardium [409]. This reduction in blood supply can manifest initially as stable angina, with patients suffering from transient chest pain during exertion. The altogether more serious manifestation of CAD, however, is acute myocardial infarction. Here, the fibrous cap on the plaque becomes displaced or eroded, exposing the prothrombotic region in the plaque core to the blood, thus inducing clot formation which can fully occlude the culprit artery. This immediate cessation of blood supply renders the myocardium ischaemic, leading eventually to cell death or infarction. The myocytes are terminally differentiated cells and do not replicate, thus once dead are not replaced. Inflammatory mediators infiltrate the infarcted myocardium and replace dead cardiomyocytes with collagen, leading to formation of a scar and, crucially, loss of contractility. Cardiac muscle efficiency is therefore compromised and this can lead to the development of heart failure.

The formation of an atherosclerotic plaque occurs over several decades, with fatty streak lesions detectable in coronary arteries as early as the second decade of life. Given the median age of first acute MI in men is estimated to be 56 years, the long incubation period for plaque formation is clear, as is the opportunity to limit its growth via avoiding its associated risk factors. Lifestyle choices such as smoking, poor diet, insufficient exercise, obesity and alcohol consumption, in addition to disease states such as diabetes, hypertension and hyperlipidaemia can significantly increase the rate of plaque formation and rupture. Indeed, it was estimated the above risk factors explain more than 95% of the population attributable risk of acute MI among men and women [8].

CAD was responsible for over 7 million deaths worldwide in 2008, 12.7% of global mortality [94]. Overall age-standardised mortality has decreased since the 1980s, particularly in high-income countries. This is attributed to decreased exposure to risk factors (e.g. smoking) and improved treatments (e.g. primary angioplasty). Lower- and middle-income countries display higher mortality to CAD, due to continued exposure to risk factors and poor prevention strategies (e.g. control of hypertension). Thus, in combination with population growth and aging, deaths from CAD continue to increase, and exert a huge economic burden on Western civilisation.

There exists a need, therefore, to develop cardioprotective interventions that can limit the extent of infarction. Indeed, over the past 20 years a huge portion of cardiovascular research has been dedicated to finding such an intervention, with a multitude of pharmacological and non-pharmacological strategies developed in both pre-clinical and clinical research. This PhD focuses on one such intervention, namely Remote Ischaemic Conditioning [283], which is promising in terms of its translational potential.

## **1.2 Myocardial ischaemia-reperfusion injury**

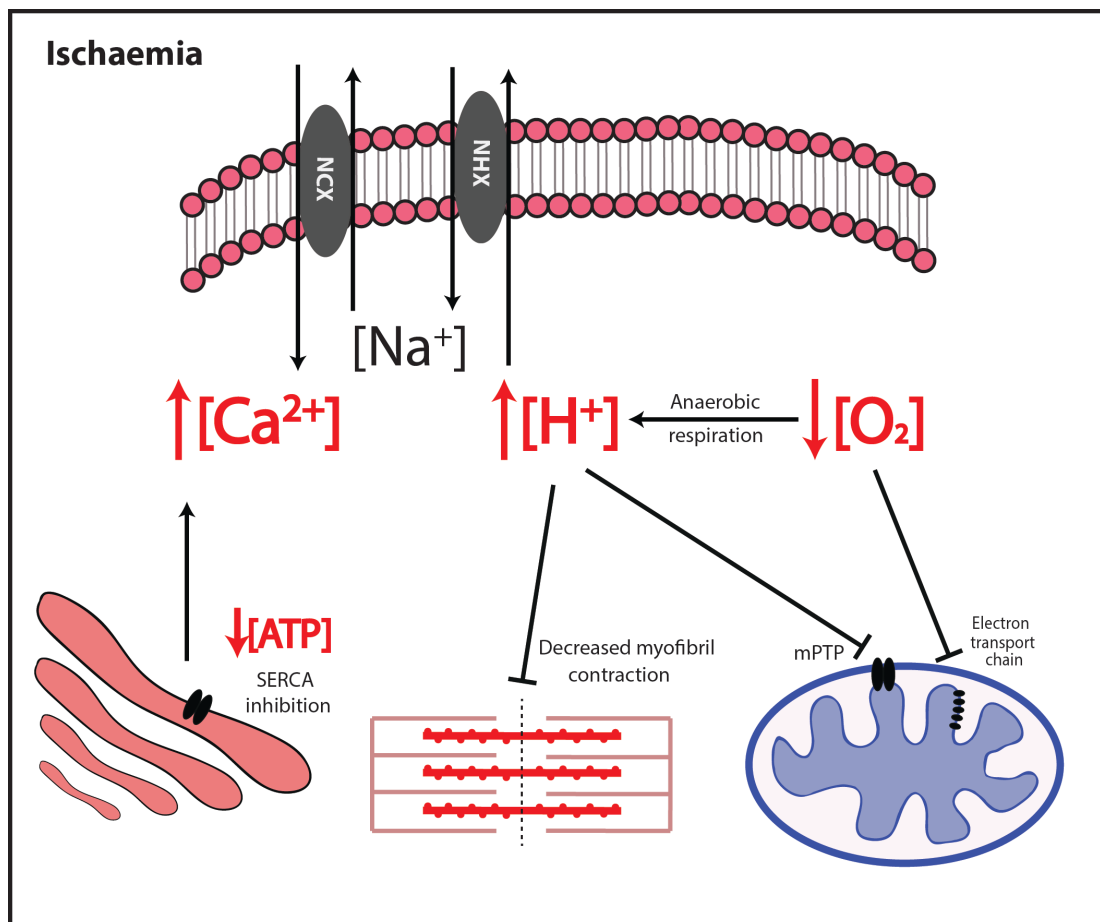
Ischaemia triggers injurious metabolic and ionic changes within the myocyte, driven primarily via the lack of glucose and oxygen, which build up over time and eventually overwhelm the cell triggering death [133]. The myocardium must therefore be reperfused before this point, to restore glucose and oxygen within cardiomyocytes and provide a chance for recovery. This reflow of blood, however, may actually extend tissue injury via release of reactive oxygen species (ROS) from the mitochondria. Termed lethal reperfusion injury, it is thought to account for up to a significant proportion of the infarction associated with IRI [389]. Indeed, reperfusion injury has been the subject of a huge volume of basic and clinical studies over the last 30 years, and several strategies have been developed which are able to limit reperfusion injury and thus reduce infarction. These interventions, along with the cellular events that occur during both ischaemia and reperfusion, are discussed below.



### 1.2.1 Ischaemic Injury

The developing ischaemic injury is governed by a tissue hypoxia, meaning the myocyte can no longer generate ATP through oxidative phosphorylation. The high energy demand of the myocyte quickly exceeds supply, resulting in a rapidly depleting supply of high energy phosphate (HEP), namely adenosine triphosphate (ATP) and creatine phosphate (CP) [303]. Notably, reduced activity of the mitochondrial electron transport chain will cause the membrane potential ( $\Delta\psi_m$ ) to subside due to accumulation of protons within the mitochondrial matrix. As a result, the  $F_1F_0$  ATP synthase, which normally produces ATP, will reverse to an ATPase in an attempt to maintain the  $\Delta\psi_m$  [163, 305]. This process is thought to account for 35-50% of ATP depletion during ischaemia, although the literature varies according to species [304]. Indeed, following 15 minutes of ischaemia the level of ATP is depleted by up to 65%, and by up to 90% after 30 minutes [162]. With  $\leq 15$  minutes of ischaemia inducing fully reversible injury and  $\geq 30$  minutes inducing irreversible injury, it seems the ATP depletion is closely related to the extent of damage within the myocyte.

The absence of oxygen will force the myocyte to produce ATP through anaerobic glycolysis. In addition to being a less efficient use of substrate, the by-product of lactate and protons will cause intracellular acidosis, with the pH decreasing to 5.5-6 during ischaemia [214]. The accumulation of protons is exacerbated by ATP hydrolysis, which releases inorganic phosphate and protons. This progressive acidosis of the cytosol and mitochondrial matrix will interact with several intracellular signalling pathways including; (1) inhibition of glycolytic enzymes that will further reduce the inefficiency of ATP production; (2) inhibition of  $Ca^{2+}$  uptake into the sarcoplasmic reticulum, contributing to the calcium overload during ischaemia [89, 183]; (3) decreased myofibril contraction, which leads to negative inotropy.



**Figure 1-1. Representative schematic of key cellular events in the cardiomyocyte during ischaemia.**

In addition to intracellular acidosis, the increase in protons within the cytosol will indirectly cause an increase in cytosolic Ca<sup>2+</sup> via a phenomenon called the coupled-exchanger theory [6]. The Na<sup>+</sup>/H<sup>+</sup> exchanger (NHE) will extrude protons from the cell while pumping Na<sup>+</sup> into the cytosol in return. With the 3Na<sup>+</sup>/2K<sup>+</sup> ATPase rendered useless due to the lack of ATP, the developing ischaemia causes a Na<sup>+</sup> overload within the myocyte. In response, the 2Na<sup>+</sup>/Ca<sup>2+</sup> exchanger will reverse, causing intracellular Ca<sup>2+</sup> overload as the ion gradients favour extrusion of Na<sup>+</sup>.

The combined effects of intracellular acidosis, ATP depletion and Ca<sup>2+</sup> overload will cause a developing ischaemic injury within the myocyte which, if prolonged for greater than 20 minutes, will induce irreversible cardiomyocyte injury and death [162, 280]. Indeed, a “wave front” of ischaemic injury occurs

which begins in the sub-endocardium and extends transmurally through the ventricles over time towards the epicardium [292].

### 1.2.2 Reperfusion Injury

Timely reperfusion is an absolute prerequisite for the survival of ischaemic tissue. Indeed, clinical implementation of early reperfusion in patients suffering from acute ST-elevation myocardial infarction (STEMI), induced via either thrombolysis or coronary angioplasty, has caused a decrease in 30-day mortality from 10.5% to 7.8% over the past 10-12 years [387]. In addition to these obvious beneficial effects, however, reperfusion can exert independent injury to the myocardium, which can lead to myocyte death [389]. Termed reperfusion injury, there are four known types as outlined below.

- i. *Reperfusion arrhythmias.* An ischaemic time less than 5 minutes is associated with reperfusion arrhythmias but does not lead to cardiac cell death [31]. These arrhythmias are self-terminating or easily treated in the clinic.
- ii. *Myocardial stunning.* When the ischaemic period extends from 5 to 20 minutes, reperfusion will induce a prolonged ventricular contractile dysfunction. This state is known as myocardial stunning and again does not induce myocyte death; the heart will fully recover its function within several days. This transient injury is thought to be mediated via a release of reactive oxygen species (ROS), with a seminal study from Bolli et al. demonstrating antioxidant therapy abolishes reperfusion arrhythmias [39]. Repeated periods of myocardial stunning, however, can lead to chronic LV dysfunction as exemplified in patients receiving dialysis [50].
- iii. *Lethal reperfusion injury.* The final and most severe level of injury occurs when the ischaemic time is over 20 minutes, where irreversible injury occurs to some of the hearts cells, resulting in infarction [84, 280]. The pathophysiology of such injury is discussed in detail in section 1.2.2.2.

- iv. *Microvascular obstruction (MVO)*. Even after reperfusion of the previously ischaemic myocardium, blood flow may still be impeded, a phenomenon known as no reflow [28, 192, 204]. This residual microvascular obstruction is due to capillary compression by endothelial cell swelling, intraluminal protrusions, platelet and fibrin microthrombi, and pericyte death [117, 193, 260, 291]. The presence of MVO is correlated with ischaemic time, but its effects are not limited to ischaemic injury. Indeed, reperfusion induces microvascular injury, which can reduce blood flow to areas that initially received adequate perfusion [7]. Clinically, it is associated with a larger infarct size, a lower left ventricular ejection fraction (LVEF), adverse LV remodelling and a worse clinical outcome [118, 356, 376, 377].

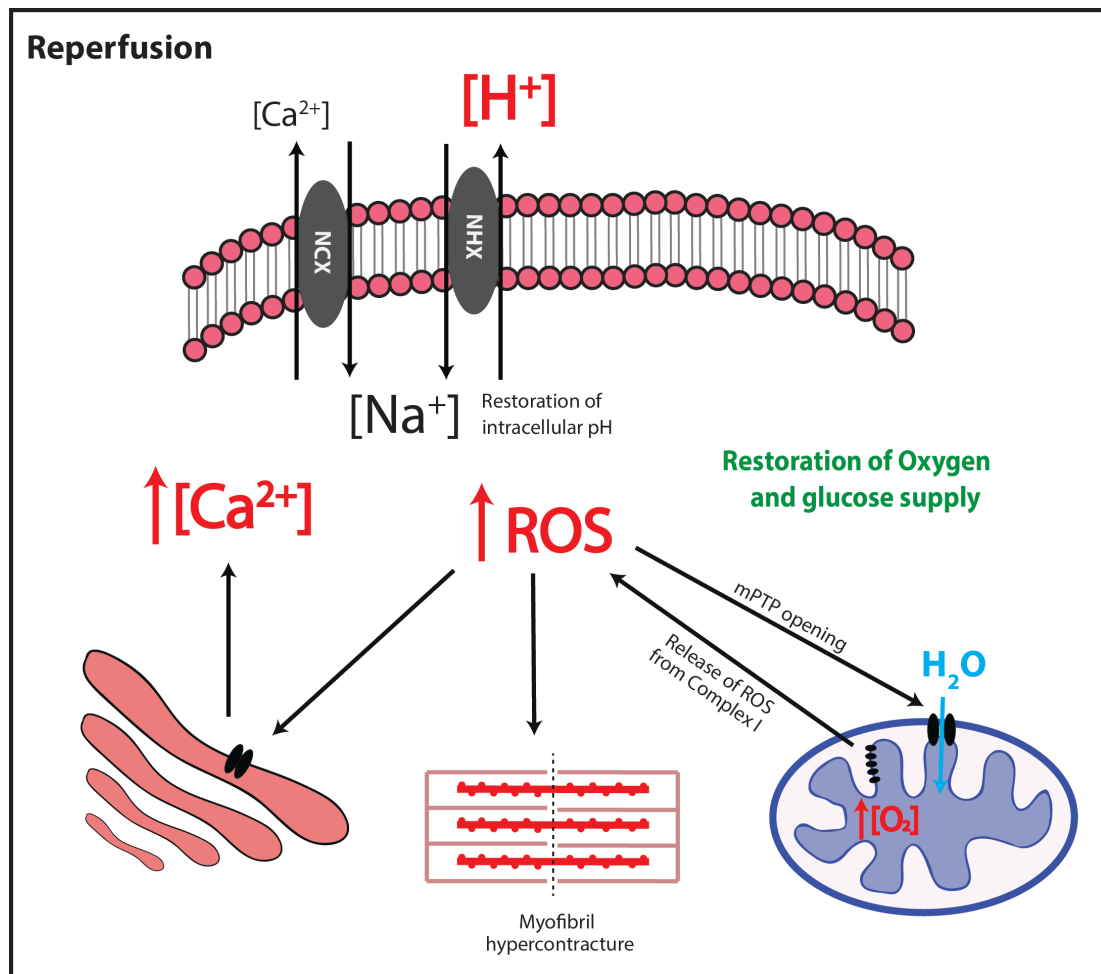
#### ***1.2.2.1 Pathophysiology of lethal reperfusion injury***

Myocardial lethal reperfusion injury is characterised by contraction band necrosis, loss of ionic homeostasis, depletion of HEP and release of intracellular enzymes. The cellular events that govern these effects include calcium overload, oxidative stress and cell swelling; these are discussed below (Figure 1-2).

##### ***Calcium overload and the pH paradox***

During ischaemia, myocytes will become loaded with  $H^+$ ,  $Na^+$  and  $Ca^{2+}$  through anaerobic glycolysis and the coupled-exchanger theory (see 1.2.1) [6]. The reintroduction of oxygen and nutrients on reperfusion will, in addition to restoring ATP production via oxidative phosphorylation, cause a rapid shift in the extracellular ionic composition, notably inducing a large proton gradient across the membrane. Thus, the intracellular acidic pH is rapidly corrected via the NHE, with the resultant  $Na^+$  overload in the cytosol causing a further influx of  $Ca^{2+}$  primarily through the NCX. A large, uncontrolled influx of  $Ca^{2+}$  can induce significant and irreversible injury to the cell. The paradoxical observation arises, therefore, that if one can delay the restoration of normal pH within the cardiomyocyte for an initial period of reperfusion, protection

may be observed via decreased  $\text{Ca}^{2+}$  overload. Indeed, several publications have suggested the existence of such a 'pH paradox' [214, 264].



**Figure 1-2 Representative schematic of the cellular sequelae of reperfusion injury.**

The large calcium overload that occurs at reperfusion cannot be handled in a normal way and results in abnormal cell function. Three major effects of calcium overload are discussed below:

- i. *Hypercontracture.* In addition to increased availability of ATP, the raised  $[\text{Ca}^{2+}]_i$  will initiate strong hypercontracture. Apart from its effect on heart function, this will cause significant physical stress to the myocyte and can contribute towards cell death [98, 297].
- ii. *Increased myocyte fragility.* Experimental evidence has demonstrated the increased  $[\text{Ca}^{2+}]_i$  can induce inappropriate activation of calpains, a

family of proteases, that can induce major physical disruption to the myocyte via weakening the cytoskeletal framework.

- iii. *Mitochondrial dysfunction.* A major focal point of reperfusion injury is the mitochondrial permeability transition pore (mPTP). An elusive channel which, when formed, spans the inner and outer membrane and abolishes the highly electronegative (-180mV) mitochondrial membrane potential ( $\Delta\Psi_m$ ). This will uncouple the respiratory chain, and may cause the outer membrane to burst and release cytochrome C, initiating cell death pathways. Calcium overload at reperfusion can increase the open probability of the pore, thus increasing the opportunity for irreversible injury.

#### *Reactive oxygen species in reperfusion injury*

Reactive oxygen species (ROS) are chemically reactive molecules containing oxygen with an unpaired electron in its outermost shell. These will very quickly react with a number of intracellular components to achieve a more stable configuration [10]. The production of ROS is not limited to purely detrimental effects in the myocyte, but plays a role in many essential signalling pathways. Indeed, the potentially damaging effects of ROS are mitigated under normal conditions by the action of superoxide dismutase (SOD) enzymes, which convert superoxide to hydrogen peroxide. A burst of ROS occurs within the myocyte during the first few minutes of reperfusion, which overwhelms the SODs and thus can induce significant injury within the myocyte [328, 408].

The primary source of ROS during reperfusion is electron leak from the respiratory chain complexes, in particular from complex I and III [60, 213, 274]. Traditionally seen as a non-specific effect of the interaction between a dysfunctional respiratory chain and the resupply of oxygen at reperfusion, recent evidence has suggested a specific metabolic process governs the production of ROS during IR. Namely, accumulation of succinate during

ischaemia that, upon reperfusion, indirectly causes extensive ROS generation from complex I [61, 271]. Indeed, pharmacological antagonism of complex I abrogates ROS production during reperfusion and limits infarct size [59]. ROS mediates reperfusion injury through lipid peroxidation leading to disruption of the SR and sarcolemma [265], mitochondrial DNA oxidation [38], protein oxidation leading to cross-linking and eventual breakdown of essential proteins, and increased open probability of the mPTP.

### *Cell swelling*

During prolonged ischaemia, the switch to anaerobic respiration for ATP production leads to the accumulation of metabolites in the cytosol, in particular lactate, which causes an increased osmotic pressure within the myocyte. The osmotic gradient created by reperfusion will result in water influx into the myocyte with resultant cell swelling. Depending on the degree of swelling, this can induce substantial physical stress and, in some cases, cell death in the absence of hypercontracture or ROS production.

## **1.3 Cardioprotective strategies**

Given the significant and irreversible injury afforded to the myocardium by IRI, a great deal of research has focussed on understanding how the heart can be protected from such lethality. Indeed, as our understanding of the mechanisms of IR-induced injury grew, many cardioprotective interventions were developed. Perhaps the most important discovery was that of ischaemic preconditioning [255]. Like most breakthrough scientific findings, it had a relatively serendipitous origin, but it formed the bedrock of a research field now in its 30<sup>th</sup> year. Not only did it prove cardioprotection was possible, but it provided a highly reproducible positive control that enabled research groups around the world to investigate the mechanisms of ischaemia-reperfusion injury, and develop new cardioprotective interventions.

### **1.3.1 Ischaemic Conditioning (IC)**

In 1986, Murry et al. published a seminal study demonstrating short bursts of non-injurious ischaemia and reperfusion rendered the myocardium significantly protected from a subsequent sustained ischaemic insult. Termed ischaemic preconditioning (IPC), this paradoxical finding was understandably met with some consternation. This soon dissolved, however, when the remarkable reproducibility of IPC was observed by hundreds of researchers across many different models of IRI from isolated cardiomyocytes and isolated perfused hearts to in vivo preparations in many species (rat, mouse, dog, rabbit and pig). The paradigm was extended by the group of Vinten-Johansen, with the finding that similar brief periods of ischaemia and reperfusion at the immediate onset of reperfusion, following prolonged ischaemia, had a similar cardioprotective effect to IPC [404]. Named ischaemic postconditioning (IPostC), this phenomenon gave convincing evidence that injury occurring at reperfusion was targetable.

The interesting aspects to IC are threefold; first, the idea that a threshold must be reached in order to achieve cardioprotection, second, the window of protection offered by IPC and, third, the extent of ischaemic injury protectable by IPC. A typical protocol would consist of 2 to 4 cycles of 2-5 minutes ischaemia with intermittent 2-5 minute reperfusion. Protection has been observed, however, with a single ischaemic episode as short as 60-seconds and, unsurprisingly, an ischaemic episode longer than 15 minutes does not induce protection. Thus, the optimum protocol lies somewhere between these numbers, and increasing the cycles is thought to increase the strength of the stimulus. Indeed, this is highlighted with the observation that a diabetic rat can be protected by three, but not one, cycles of brief ischaemia and reperfusion [372]. A typical IPostC protocol consists of 3 cycles of 30 seconds reperfusion followed by 30 seconds of coronary reocclusion at the immediate onset of reperfusion from the injurious ischaemia [404].

It is worth noting, indeed, that the field has not been unanimous with its acceptance of IPostC, with several studies suggesting neutral effects of the



intervention [82, 314]. Moreover, whilst several clinical studies have demonstrated positive effects of IPostC on myocardial IRI [229, 344], others have shown only a modest or neutral effect [97, 225, 329, 337].

The standard interval between the end of the IPC protocol and the induction of prolonged ischaemia is between 5 and 15 minutes, however the protective conditioning lasts for between 1 and 4 hours following the stimulus depending on the duration of the injurious ischaemia [113]. The protection then wanes but, remarkably, re-emerges 24 hours later in a phenomenon known as 'second window' of protection (SWOP) [388]. Conversely, IPostC was initially thought to be protective if performed within only the first few minutes of reperfusion, however it been claimed to remain effective even when performed as late as 30 minutes following reperfusion [23, 302]. Interestingly, the cardioprotective mechanism of this delayed IPostC seems to be distinct from that of early IPostC and IPC [23].

Finally, a critical feature of IPC is the strength of protection is dependent on the length of index ischaemia. Indeed, in models of permanent coronary artery occlusion, or when the sustained ischaemia is prolonged to >2 hours, IPC fails to limit infarct size [255]. Thus, the necessity for timely reperfusion is clear. IPostC has a more complex relationship with index ischaemia, with a bell-shaped curve of protection. An ischaemic time of >60 minutes mutes the protection afforded by IPostC [239]. When this is reduced to 45 minutes, however, powerful protection is observed. Unexpectedly, when the index ischaemia is <30minutes, IPostC seems to give neutral or even detrimental results [239, 272, 314].

Initially, the cardioprotection afforded by IPC was attributed to metabolic adjustments within the cell, in particular a decreased rate of energy metabolism [256]. Whilst this is undoubtedly involved in the mechanism, IPC seems to be initiated via receptor binding. A landmark study by Liu et al demonstrated adenosine receptor activation was fundamental to inducing cardioprotection [226]. Indeed, there is no question that IPC is a receptor-mediated phenomenon, via release of one or more trigger molecules, notably

adenosine, opioids and bradykinin [reviewed in 43]. These will bind to their respective G-protein-coupled receptors (GPCR) on the sarcolemma and trigger a complex and diverse array of intracellular signalling pathways, which leads to attenuation of the injury associated with ischaemia and reperfusion. Whilst IPC and IPostC seem to exert cardioprotection via similar mechanisms, IPC by definition induces the cardioprotective state prior to IR. Here, IPC is distinct as it involves a memory component induced via the trigger(s) that must last until the onset of the lethal ischaemia and, crucially, until reperfusion to exert its protection via several effector pathways [129]. With IPostC, however, the trigger, mediator and effector steps must all occur both speedily and almost simultaneously.

- i. *Trigger phase (IPC only)*. The brief cycles of ischaemia induce release of several small molecules, including adenosine [226], opioids [74, 268] and bradykinin [105]. These bind to and activate their respective receptors, leading to activation of an intracellular signalling pathway, which includes epidermal growth factor receptor (EGFR), phosphoinositide-3 (PI3) kinase and Akt, nitric oxide synthase (NOS) and protein kinase G (PKG). In addition, a recent publication including data presented in this thesis (Chapter 6) has implicated intrinsic cardiac ganglia in the trigger pathway [277]. These various signalling mechanisms converge on the activation of mitochondrial ATP sensitive potassium channels (mitoK<sub>ATP</sub>), resulting in a small increase in production of ROS. These ROS are thought to activate, via oxidation, several cardioprotective kinases, notably protein kinase C (PKC), thus inducing the memory state associated with IPC. Although PKC is thought to be the primary source of the memory component, there seem to be species-specific differences in the importance of the kinase in IPC [reviewed in 21 & 42]. In general, the exact mechanism of the memory component is not yet clear.
- ii. *Effector phase (IPC and IPostC)*. In the first few minutes of reperfusion, adenosine receptor activation occurs which, perhaps via PKC, induces activation of the reperfusion injury salvage kinase (RISK) pathway. This pathway, pioneered by the Hatter

Cardiovascular Institute, describes a group of kinases whose activation is associated with cell survival. For example, activation of PI3-kinase, Akt and extracellular-related kinase (ERK) at reperfusion is a fundamental process in IPC-mediated cardioprotection [130, 131, 251, 396]. Other signal pathways, such as the survival activating factor enhancement (SAFE) pathway, involving activation of tissue necrosis factor 1 alpha (TNF-1 $\alpha$ ) and intracellular kinases such as janus kinase (JAK) and STAT-3, have also been shown to play a role [210, 212, 333]. This constellation of receptor-mediated signals is proposed to converge on the mitochondria and, perhaps via phosphorylation of GSK-3 $\beta$ , prevent the swelling and rupture by inhibiting the formation of the permeability transition pore (mPTP) [120, 123]. Preventing mitochondrial rupture will significantly shift the balance of the cell towards survival during IR.

### **1.3.2 Remote Ischaemic Conditioning**

The major disadvantage of IPC and IPost, from a clinical perspective, is the requirement to apply an invasive intervention to the heart directly. In this regard, the phenomenon of remote ischaemic conditioning (RIC), which allows the protective stimulus to be applied to an organ or tissue away from the heart, has the upper hand.

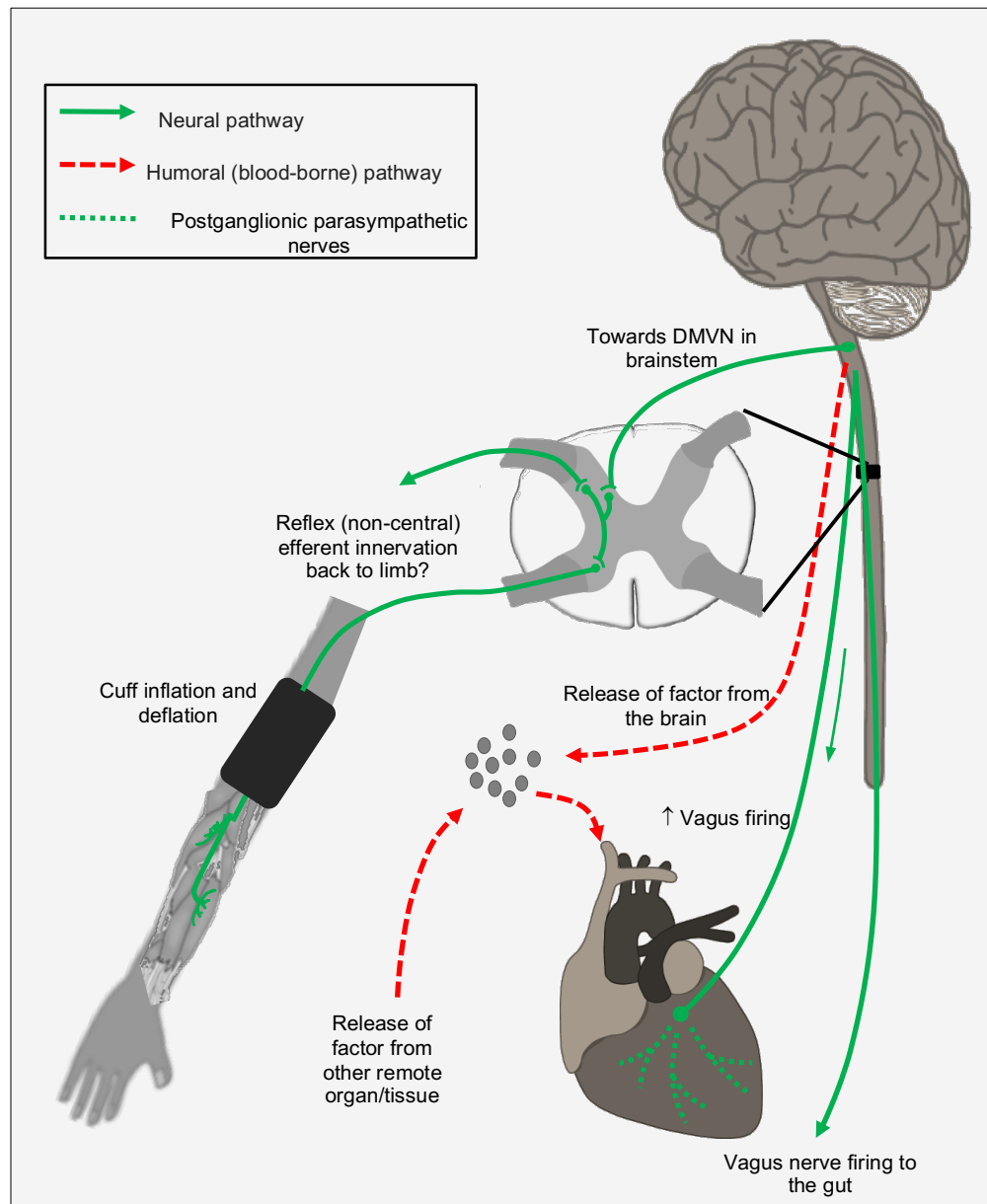
The experimental study to first describe this cardioprotective phenomenon was published by Przyklenk et al in 1993 [283]. In the canine heart, applying four 5 min cycles of occlusion and reflow to the circumflex coronary artery was found to reduce infarct size induced by 45 minute occlusion and 3 hours reperfusion of the left anterior descending (LAD) artery, suggesting that cardioprotection could be transferred from one coronary territory to another. This transfer of cardioprotection was then extended to a remote organ, the kidney, by Gho et al, who demonstrated that 15 minutes of occlusion and reflow in the mesenteric artery, could reduce infarct size induced by 60 minutes ligation and 3 hours reperfusion of the left main coronary artery [101], extending the paradigm from intra-cardiac to inter-organ protection. Subsequent experimental studies have reported that the preconditioning stimulus could be applied to a number of different organs remote from the heart including the intestine, liver and brain. However, in terms of facilitating the translation of RIC into the clinical setting, the major advance was made by Birnbaum et al, who discovered that the RIC stimulus could be applied to the gastrocnemius muscle of the hind-limb by partially occluding the femoral artery [35]. Furthermore, Oxman et al. demonstrated that the conditioning stimulus could be applied non-invasively using a tourniquet applied to the hind-limb [263]. The discovery that RIC could be elicited non-invasively, by simply inflating and deflating a cuff placed on the upper arm or leg in human volunteers, greatly facilitated its translation into the clinical setting.

One other major advantage of RIC is its ability to confer cardioprotection when applied at a number of different time-points in relation to the index myocardial ischaemia-reperfusion episode, a feature that has also facilitated

its clinical application. The early experimental studies focused on applying the conditioning stimulus immediately prior to the index myocardial ischaemic episode (remote ischaemic **PRE**conditioning or RIPC [35, 283]), with subsequent experimental studies reporting efficacy with the conditioning stimulus applied at varying time-points: 12-24 hours prior to the index myocardial ischaemic episode (delayed remote ischaemic **PRE**conditioning) [231, 405]; after the onset of myocardial ischaemia but prior to reperfusion (remote ischaemic **PER**conditioning) [311]; at the onset of myocardial reperfusion (remote ischaemic **POST**conditioning) [9]; and most recently, even after 15 min of myocardial reperfusion has elapsed (delayed remote ischaemic **POST**conditioning) [23]. Interestingly, repeated remote post-conditioning applied once per day for 27 days post-infarction drastically improves survival and cardiac remodelling [364].

### **1.3.3 Mechanisms underlying RIC**

The actual mechanistic pathway underlying RIC-induced cardioprotection remains unclear, but it can be divided in three inter-related events: (a) generation of the cardioprotective signal in the conditioned remote organ or tissue; (b) the pathway which conveys the cardioprotective signal from the conditioned remote organ or tissue to the heart; and (c) the activation of intracellular signalling pathways within the heart which mediate the cardioprotective effect (Figure 1). The current paradigm dictates there are both neural and humoral aspects to (a) and (b), their involvement and potential interdependence are discussed below.



**Figure 1-3: Schematic describing the possible mechanisms of communication of RIC.** Brief periods of ischaemia-reperfusion to the limb, via cuff inflation/deflation, causes local autocoid release. This in turn activates sensory afferent neurones which relay, via the spinal cord, to the DMVN in the CNS. Activation of nuclei within the DMVN results in increased vagal nerve firing to the heart which, via release of ACh and subsequent activation of mAChRs, induces the cardioprotective phenotype. In addition, following activation of afferent sensory neurones in the conditioned limb, there is release of a dialysable cardioprotective factor into systemic circulation. The source of this factor remains unknown, although possibilities include i) from the conditioned limb itself ii) from the central nervous system iii) from pre-/post-ganglionic parasympathetic nerve endings within the heart iv) from a non-conditioned remote organ/tissue.

### **1.3.3.1 Blood-borne cardioprotective factor (s)**

A number of experimental studies have implicated a blood-borne factor(s) generated in the RIC-treated animal as potential mediators of cardioprotection. The evidence for this includes the following:

1. Gho et al [101] noted that RIC applied to the intestine would only protect the heart if it was reperfused prior to coronary occlusion, suggesting that reperfusion was required to wash the cardioprotective factor out of the conditioned tissue.
2. Dickson et al [75, 77] discovered that the ischaemic preconditioning effect could be transferred from one rabbit to another non-preconditioned rabbit, first via whole blood transfusion, and secondly via transfer of coronary effluent from a preconditioned to a naïve isolated heart. Hence, the presence and necessity of a blood-borne cardioprotective factor is clear. This model has been reproduced across several species [43, 215, 317, 320] and has played a central role in recent characterisation of the humoral factor.
3. Preliminary evidence suggested an intact opioid receptor system was required for protection to occur [74, 320], however opioid levels were not raised in IPC effluent [74]. A subsequent study [215] demonstrated raised adenosine levels in IPC effluent and, via use of the adenosine receptor blocker, 8-(-p-sulfophenyl)theophylline (8-SPT), that adenosine receptor activation was required for effluent-mediated protection. Combined together, this evidence could suggest adenosine and opioid receptor crosstalk [266, 334].
4. Current evidence suggests the factor is between 3.5 kDa and 30 kDa, thermolabile and hydrophobic. Using dialysis membranes to fractionate the proteins within coronary effluent or serum according to their molecular weight, several studies demonstrated the factor to be smaller than 30 kDa [43, 278, 320, 331] and, crucially, larger than 3.5 kDa [317]. This would suggest small molecules, such as adenosine and opioids, are not essential for RIC (adenosine 267.24 Da, opioids 500-800 Da, bradykinin 1060.22 Da). Moreover, using a series of chromatographic and heating

steps, it was proposed that the factor was both thermolabile and hydrophobic, indicating it may be a protein [43, 317].

5. Proteomic analysis of plasma, via a combination of two-dimensional gel electrophoresis (2D-DIGE) and mass spectrometry (MS), unmasked an altered plasma proteome following RIC. Studies by Lang, Hepponstall and Hibert found 4, 51, and 30 differentially expressed proteins in RIC plasma relative to control respectively [139, 143, 207]. These proteins, linked to the regulation of various cellular functions, including the acute phase response, immune response, haemostasis and lipid transport, suggested a complex interaction of signalling pathways in response to RIC. Broad proteomic analyses, however, are not well suited to the hypothesis of a single, small, low abundance protein conveying the cardioprotection, due to the large number of high abundance proteins in plasma. Indeed, a recent proteomic study in 6 human subjects saw no significant upregulation of proteins in plasma following RIC [138]. With this in mind, two studies have analysed coronary effluent using liquid chromatography (LC) and MS, the advantage being the majority of plasma proteins (high abundance) are removed [197, 215]. Initial investigation revealed 185 unique proteins in coronary effluent following five cycles of 5 minutes global ischaemia and 11 minutes reperfusion in an isolated rat heart. Only 30.3% were plasma proteins, with the remainder originating from the cytoplasm and various intracellular organelles. A subsequent publication alluded in the discussion to a proteomic (LC-MS) analysis of coronary effluent from isolated rat hearts, which found 8-10 peptides were increased by >50% relative to control effluent, and ~100 uncharacterised ions corresponding to metabolites or proteins [215]. Thus, although high abundance proteins are largely excluded in coronary effluent, a significant amount of protein is still released, indicating that effluent must be further fractionated before MS if a potential biomarker is to be found.
6. Several experimental studies have suggested that the cardioprotective state elicited by an RIC stimulus delivered *in vivo* remained, even when the heart was isolated and subjected to acute MI either on a Langendorff-apparatus [203] or in a transplanted recipient animal [196]. These findings suggest that an intact neural pathway to the heart was not required for



RIC-cardioprotection during the acute MI, but it does not of course exclude the need for an intact neural pathway at the time of the RIC stimulus.

7. Although the identity of the blood-borne cardioprotective mediator of RIC remains unknown, a number of candidate molecules have been suggested including opioid [74], adenosine [215], bradykinin [312], erythropoietin, calcitonin-gene related peptide, stromal derived factor 1- $\alpha$  (SDF1- $\alpha$ ) [72], nitrites [287] and micro ribonucleic acid 144/451 cluster (miRNA 144/451) [218].

#### **1.3.3.2 Neural pathway**

A number of experimental studies have suggested the involvement of a neural pathway in RIC cardioprotection. The evidence for this is summarised below:

1. Gho et al [101], first noted that the ganglion blocker, hexamethonium, blocked RIC-induced cardioprotection elicited by intestinal conditioning, thereby implicating a role for the autonomic ganglia of the sympathetic and parasympathetic nervous systems in inter-organ protection. A subsequent study confirmed in human volunteers that trimethaphan, an autonomic ganglionic blocker, abrogated limb RIC protection of endothelial function [231]. Two studies, however, did not see any abolition of RIC with hexamethonium [188, 367], suggesting further investigation is perhaps necessary.
2. Given the involvement of the autonomic nervous system in RIC, it is logical to hypothesise that RIC cardioprotection is dependent on an intact sensory afferent neuronal pathway at the remote organ or tissue. Indeed, transection of the femoral nerve before application of the RIC stimulus abolishes cardioprotection [223, 331]. Moreover, Ding et al. noted that brief renal artery occlusion (RAO) resulted in increased afferent renal nerve activity (ARNA), and its transection also abolished RIC-induced cardioprotection [78]. Similarly, direct stimulation of the sensory nerve of

the remote organ or tissue has been reported to recapitulate the cardioprotective effect elicited by RIC [81, 83, 248, 288]. Finally, stimulation of cutaneous sensory nerves using either topical application of capsaicin [290] or surgical skin incision (see later section on remote preconditioning of trauma, RPCT) [112, 293] has been reported to also mimic RIC cardioprotection. One recent study, however, demonstrated femoral nerve section did not abolish hindlimb RIC in the mouse, thus there remains contention as to the exact role of the sensory nerve in the RIC trigger pathway [287].

3. The question arose, therefore, of how the brief ischaemic burden to the tissue or organ is translated to sensory afferent activation. Experimental studies have demonstrated that the application of brief ischaemia/reperfusion to a remote organ or tissue generates factors such as adenosine [78, 222, 270], bradykinin [312] and calcitonin gene related peptide (CGRP) [48] in the remote organ or tissue which then stimulate the local sensory neural afferent pathway. Indeed, the adenosine receptor antagonist 8-SPT abrogated the increased ARNA observed by Ding et al. following brief RAO [78]. In addition, intrafemoral-arterial injection of adenosine, whilst not at a level sufficient to elicit cardioprotection alone, was able to induce cardioprotection via a mechanism requiring an intact sciatic nerve [331]. Thus, it seems RIC induces local release of a neuro-active factor, which in turn activates sensory afferent neurones and initiates the cardioprotective message.
4. The final element concerns the efferent limb of the neural pathway to the heart. Initial studies demonstrated that vagal nerve stimulation (VNS) mimicked the cardioprotective effect of RIC, whereas with bilateral vagotomy this was abrogated [51, 79, 174]. An elegant study by Mastitskaya et al demonstrated, using optogenetics, that activation of the dorsal motor nucleus of the vagus nerve (DMVN) was sufficient to induce cardioprotection [243]. Moreover, this effect was abrogated in the presence of atropine. Two papers, however, counteract this paradigm; Firstly, spinal cord transection at C7, thereby removing central nervous innervation, did not abolish RPCT (see section 1.3.3.5) [167]. Secondly, evidence that VNS prior to index ischaemia increases infarct size [49].

### **1.3.3.3 *Neuro-humoral pathway***

Initial experimental studies had implicated a blood-borne cardioprotective pathway and an intact neural pathway to the conditioned organ or tissue. The actual interplay between these components remains unknown, although recent studies have investigated the interaction between these two signalling components:

1. It has been reported that RIC by limb ischaemia or intra-arterial adenosine releases a dialysable blood-borne cardioprotective factor(s), the release of which required an intact sensory innervation of the limb and was blocked by pretreatment with the nitric oxide (NO) donor, S-nitroso-N-acetylpenicillamine (SNAP) [331]. Interestingly, production of NO, via activation of nitric oxide synthase, is seemingly not required for production of the cardioprotective factor but is required for induction of the protective phenotype [318].
2. An important experimental study by Redington et al demonstrated that direct femoral nerve stimulation or topical application of capsaicin generated a dialysate which was able to reduce MI size in a naïve isolated rabbit heart, providing the first evidence that the neural pathway to the conditioned limb was required to generate the blood-borne cardioprotective factor [290].
3. In an interesting clinical study, Jensen et al found that plasma dialysate obtained from RIC-treated diabetic patients with sensory neuropathy failed to limit MI size in an isolated naïve rabbit heart, although plasma dialysate harvested from RIC-treated non-diabetic and diabetic patients without sensory neuropathy was cardioprotective [164].
4. The link between sensory nerve activation and release of the humoral factor is likely the vagus nerve. A recent study from Mastitskaya et al. demonstrated that surgical section of the vagus nerve below the diaphragm abrogated in vivo RIC in rat [242]. This suggested that the humoral factor was released following vagal innervation of the gut, however this was not explicitly proven. A subsequent study from the same

group suggested that glucagon-like peptide 1 (GLP-1) was released from the gut following RIC [25]. Finally, a study published using data in this thesis added weight to these data, by demonstrating bilateral cervical vagotomy prevented release of the humoral RIC mediator [278] (see Chapter 5).

Therefore, the current paradigm suggests that the conditioned limb generates adenosine in the local circulation, which then activates the sensory neural pathway (through a mechanism dependant on nitric oxide), leading to the activation of dorsal nuclei within the brainstem. This leads to increased systemic vagal tone and subsequent release of a humoral blood-borne factor, which moves to the heart and protects [278]. This was a key finding from this PhD (See Chapter 5).

#### **1.3.3.4 Systemic response pathway**

There is some evidence, although inconsistent, that a systemic inflammatory response is obtained in the setting of reperfusion injury. Reperfusion injury *per se* is associated with a neutrophilic infiltration within the target organ [389]. Remote Ischaemic PreConditioning (RIPC) seems to have an anti-inflammatory effect. For instance, a group conducting experiment on skeletal muscle flaps noted that RIPC reduced the number of leucocytes adhering the post capillary venules [205]. Similarly, RIPC decreased overall levels of interleukin-1 $\beta$  and interleukin-6 in a porcine model of lung ischaemia-reperfusion injury. In a human forearm endothelial model of ischaemia reperfusion injury, neutrophil adhesion was reduced by RIC, as was phagocytosis [319]. Even pro inflammatory gene expression in leucocytes appears to be suppressed, while the anti-inflammatory gene expression is up regulated [195].

As mentioned above, the vagus nerve appears to play a key mechanistic role in RIC. There is evidence that vagus nerve stimulation (VNS) can induce a systemic anti-inflammatory effect [261]. One mechanism by which this occurs

is release of acetylcholine from the spleen and the gut [40]. Efferent vagal firing to the enteric nervous system will release acetylcholine at the synaptic junction with macrophages. In the spleen, efferent vagus firing activates the splenic sympathetic nerve. This in turn stimulates splenic lymphocytes to release acetylcholine. In both cases, the acetylcholine will inhibit release of tumour necrosis factor alpha (TNF $\alpha$ ), a pro-inflammatory cytokine, from macrophages [300]. Given there is a well-defined injurious inflammatory response to acute myocardial infarction, reducing systemic inflammation via VNS could be additive to the cardioprotective mechanism of RIC. Indeed, high serum TNF $\alpha$  is a well-established predictor of mortality following acute myocardial infarction [90].

However, when a panel of circulating cytokines were analysed in adults and children undergoing cardiac surgery, RIPC did not produce any change in anti-inflammatory or pro-inflammatory cytokines [58, 173]. In a similar study by Albrecht et al, circulating levels of interleukin-1 $\beta$ , interleukin-8 and tumour necrosis factor- $\alpha$  before CPB were increased in patients subjected to RIPC compared to control [3]. This pro-inflammatory response directly contradicts pre-clinical evidence and hence, the exact role of a systemic response is unclear and needs further investigation.

#### ***1.3.3.5 Remote preconditioning of 'Trauma' (RPCT)***

The concept of RPCT originally arose from the observation that patients who had undergone abdominal aortic aneurysm surgery had a greater risk for MI, a greater infarct size and increased mortality compared to those who had not undergone surgery [298, 349]. Indeed, Ren et al. observed that infarct size in mice increased with prior non-ischaemic carotid vascular surgery. However, Ren et al. subsequently demonstrated, in the same study, that a small cutaneous incision to the abdomen was able to limit MI size in a manner similar to RIC [293]. This unusual observation, termed remote preconditioning of trauma, has been demonstrated in two further studies, with the current paradigm suggesting the protection occurs via activation of A $\delta$ -

and C- sensory afferent neurons [112, 167], which confer protection to the heart perhaps independent of central innervation [167].

This compliments the theory that small diameter C-fibre afferents, which include nociceptive afferents, are responsible for communicating the cardioprotective message away from the conditioned organ/tissue. As discussed above, several different methods of activating such afferents (TENS [248], capsaicin [290], ischaemia [283], surgical trauma [293]) all lead to cardioprotection. Perhaps the commonality between these interventions is the C-fibre sensory afferent nerve.

#### **1.3.4 Which aspects of the RIC mechanism remain elusive?**

In pre-clinical literature, the fundamental questions of the mechanisms of RIC have not been adequately answered. Indeed, there is a distinct lack of studies that have systematically characterised RIC, particularly in terms of the optimal intervention protocol. For example, what are the relative benefits of 1 cycle when compared to 3 cycles? Is RIC more effective when applied to one or more limbs? Although a recent publication and meta-analysis attempt to address these issues, further research is still required [166, 368]. Moreover, our recent meta-analysis identified only 31 studies investigating RIC in animal in vivo models of myocardial infarction since 1997 ([45] and Chapter 4). This is a very small number, particularly when compared to the 477 studies investigating classical ischaemic conditioning published in the same time period [368]. This is perhaps the main reason for our current lack of understanding. The paucity of pre-clinical RIC research means there are several aspects to the mechanism that remain hidden. The presence and necessity of a humoral factor is clear, as is the dependence on intact sensory innervation to the conditioned limb for its production. We and others have presented a key role for the vagus nerve in RIC, its activation being required for subsequent release of the humoral mediator. However, the exact source and identity of the blood-borne cardioprotective factor remains unknown. The absence of a validated biomarker for RIC significantly hinders one's ability to

interpret neutral data. Thus, identification of this factor remains the Holy Grail for pre-clinical RIC research.

#### **1.4 Clinical translation of RIC**

The non-invasive and low-cost nature of RIC lends itself well to clinical translation [323]. The first setting investigated was cardiac surgery, including valve replacement and coronary artery bypass surgery. Here, RIC could be easily applied to the patient whilst under anaesthesia, thus avoiding potential tolerability issues. Cheung et al. first demonstrated a protective effect of RIC in children undergoing cardiac surgery for valve replacement [58]. Four cycles of 5 min lower limb ischaemia and reperfusion reduced peri-operative injury, as measured by serum troponin I. Although several meta-analyses indicate an overall protective effect of RIC in this setting [70, 116], there are a growing number of neutral trials [149, 391]. In particular, two recent large multicentre studies demonstrated no benefit of RIC in patients undergoing coronary artery bypass surgery, as measured by 30-day [249] and 1-year [122] outcome (Table 1-1). Thus, it appears that RIC is not appropriate for use as a therapeutic agent in cardiac surgery, the reasons for which are discussed below (section 1.4.1). RIC has also been employed in the setting of elective percutaneous coronary intervention (PCI). The first indication of a positive effect came from Hoole et al., who demonstrated in 242 patients that three 5 min cycles of upper limb ischaemia-reperfusion reduced peri-procedural injury [150]. Remarkably, this protective effect was still apparent 6 years following the intervention [73]. Again, there are a mixed population of studies in the setting, with both positive and neutral results. A more successful therapeutic role for RIC is surely primary PCI in patients suffering from ST-elevation myocardial infarction (STEMI). This is the clinical setting most similar to the acute ischaemia-reperfusion injury studied in pre-clinical models. The majority of trials in this setting have indicated a protective effect of RIC, with only one recent neutral study. The ongoing ERIC-PPCI and CONDI-2 trial, recruiting 4300 patients, is probably the best chance of translating RIC to the clinic [126]. Several key clinical trials investigating RIC

are detailed in table 1, and reasons for the failed clinical trials discussed in more detail below.



Study	Patient number	Setting	RIC protocol	Study comments	Results
Cheung (2006) [58]	37	Children undergoing surgery for congenital heart defects	4 x 5 min lower limb ischaemia/reperfusion	<ul style="list-style-type: none"> <li>- Single centre single blinded study</li> <li>- Endpoints of lung mechanics, serum cytokines and troponin I</li> </ul>	<p>Decreased serum troponin I in the RIC group</p> <p>RIC group had lower airway resistance at 6hr post-operation</p> <p>RIC group had reduced inotropic requirement at 3hr and 6hr post-operation</p>
ERICCA (NCT01247545)  <i>Hausenloy et al</i> [122]	1610	CABG surgery with CPB	4 x 5 min upper limb occlusion/reperfusion	<ul style="list-style-type: none"> <li>- Multi-centre, single blinded study</li> <li>- Primary endpoint MACCE at 1 year</li> <li>- Secondary outcomes include serum cTnT AUC, LVEF%, AKI, 6 min walk test</li> </ul>	<p>No difference in primary outcome</p> <p>No difference in any secondary outcome</p>
RIPHeart  <i>Meybohm et al</i> [249]	2070	All elective cardiovascular surgery with CPB	4 x 5 min upper limb occlusion/reperfusion	<ul style="list-style-type: none"> <li>- Multicentre, double blinded study</li> <li>- Primary endpoint composite all-cause mortality over 14 days after surgery</li> </ul>	<p>No difference in primary outcome</p> <p>No difference in any secondary outcome</p>

(NCT01067703)					
Hong (2014) [148]	1280	All elective cardiac surgery on/off pump with cold blood cardioplegia	4 x 5 min upper limb occlusion/reperfusion  Both PRE- and POST-operatively	- Multi-centre trial  - Primary endpoint was composite outcome	No difference in composite primary outcome.  No difference in secondary outcome (ICU/hospital stay)
Thielmann (2013) [340]	329	Elective CABG on pump with cold crystalloid cardioplegia	3 x 5 min upper arm occlusion/reperfusion	- Single centre, double blinded  - AUC serum cTnI primary endpoint  - Mortality as main safety endpoint	No significant difference in cTnI AUC  Lower all-cause mortality at 1 year  Lower MACCE at 1 year
Carrasco-Chinchilla (2013) [54]	232	Elective PCI	3 x 5 min upper limb occlusion/reperfusion  Remote ischaemic postconditioning	- Single centre blinded study  - Serum cTnI AUC primary outcome	No difference in cTnI at 24 hours  Diabetic patients in RIC group had higher incidence of PCI-MI
Davies WR (2013) [73]	225	Elective PCI	3 x 5 min upper arm occlusion/reperfusion	- Single centre blinded study  - 6-year MACCE follow up to previous Hoole et al study	Lower MACCE at 6 years

Luo (2013) [235]	205	Elective PCI	3 x 5 min upper arm occlusion/reperfusion	<ul style="list-style-type: none"> <li>- Single centre unblinded study</li> <li>- Both single and combined targets stented</li> <li>- Serum cTnI AUC primary outcome</li> </ul>	<p>Significantly reduced cTnI at 16 hours</p> <p>No change in renal outcome</p>
Botker (2010) [41]	251	STEMI patients treated with PPCI	<p>4 x 5 min upper limb occlusion/reperfusion</p> <p>RIC started in the ambulance on confirmation of STEMI</p>	<ul style="list-style-type: none"> <li>-Single centre single blinded study</li> <li>-Primary end point was myocardial salvage index determined by SPECT†</li> </ul>	<p>Increased myocardial salvage in the RIC group</p> <p>Increased left ventricular ejection fraction at 24 hours in the RIC group but not at 30 days</p> <p>No difference in 30 day MACCE‡</p>
Crimi (2013) [68]	96	STEMI patients treated with PPCI	<p>3 x 5 min lower limb occlusion/reperfusion</p> <p>Remote ischaemic postconditioning</p>	<ul style="list-style-type: none"> <li>-Multi centre single blinded study</li> <li>- Only TIMI 0 and 1 flow patients randomised (total or near total occlusion)</li> <li>- Primary endpoint CK-MB AUC</li> </ul>	<p>Decreased CK-MB AUC in the RIC group</p> <p>Decreased myocardial oedema as determined on T2-weighted CMR§ imaging in the RIC group</p> <p>Better ST segment resolution in the RIC group</p>

Sloth (2014) [326]	251	STEMI patients treated with PPCI	4 x 5 min upper limb occlusion/reperfusion	-Follow up of MACCE to study by Botker et al  -Median 3.8 years follow up	Decreased MACCE in RIC group compared to the control group
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**Table 1-1: Key trials investigating RIC in the clinic**

Study	Patient number	Setting	RIC protocol	Study comments	Current progress
ERIC-PPCI/CONDI-2  [127]	4300	STEMI patients treated with PPCI	<p>4 x 5 min upper limb occlusion/reperfusion</p> <p>Given in the ambulance (CONDI-2) or at the hospital (ERIC-PPCI)</p> <p>Remote ischaemic preconditioning</p>	<p>- Designed originally as two separate studies, subsequently combined to increase statistical power.</p> <p>- CONDI-2 in Denmark, ERIC-PPCI in UK. Multicentre, single blinded study.</p> <p>- Primary endpoint cardiovascular fatality and hospitalisation for heart failure at 1 year</p> <p>- Secondary outcomes include serum cTnT AUC, LVEF%, AKI, 6 min walk test</p>	Recruitment of patients to study

**Table1-2: Key upcoming trial investigating RIC in the clinic**

#### **1.4.1 Why the negative clinical trials?**

A number of clinical studies have failed to find any beneficial effects of limb RIC in patients undergoing PCI [156], CABG [122, 173, 249] and vascular surgery [364] – these include some large clinical trials conducted in paediatric [246] and adult cardiac surgery [149, 285]. Several review articles have been published analysing the potential reasons underlying the failure to translate cardioprotection into the clinical setting [121, 262, 276, 315]. The biggest limitations of these clinical trials lie in their study design. The vast majority of them have been single-centre trials examining small groups of patients, in particular, admitted for elective CABG surgery. This represents less than 50% of patients undergoing major cardiac surgery in England and Wales. Moreover, most of the trials have been single-blinded, leaving room for bias. In fact, one meta-analysis suggests that single-blinded trials were mostly positive, while double-blinded ones were mostly negative [391]. Secondly, the primary end point used in almost all the trials was the area under the curve of a 48-hour or 72-hour collection of troponin or creatinine kinase (CK-MB) post-procedure. While there is evidence that higher troponin and CK-MB levels after cardiac surgery correlate with increased mortality at one year, much of this evidence comes from small single-centre, mixed prospective, retrospective and post-hoc analyses, as discussed in a meta-analysis by Petaja et al [273]. This represents a surrogate endpoint and stronger clinical endpoints such as post-operative morbidity and mortality, and patient-centred indices such as quality of life are lacking. Thirdly, many of the initial studies excluded diabetic patients, as they were proof of concept trials. Indeed, several complex high-risk patients with unstable angina, recent myocardial infarctions, renal disease and complex surgery, were excluded. Co-morbidities have been demonstrated to interfere with the efficacy of cardioprotection [92, 372, 380]. Given that the risk profile of patients undergoing cardiac surgery or PCI is becoming increasingly complex, this represents another major drawback. Finally, a major criticism of the recent large scale clinical trials in cardiac surgery is the use of propofol as an

anaesthetic agent in a very high proportion of patients. This has been documented to inhibit the efficacy of RIC [198]; interestingly, preclinical investigation demonstrated it to inhibit vagus nerve firing [361] (discussed further in Chapter 5).

#### **1.4.2 Will RIC ever change current clinical practice?**

The one setting in which the effect of RIC has been predominantly positive is in STEMI patients treated by PPCI with 4 proof-of-concept studies reporting cardioprotective effects with limb RIC applied at the time of PPCI [41, 68, 281, 294]. Indeed, the majority of the published clinical studies have established that limb RIC can limit myocardial injury in PCI, CABG and STEMI patients. A recent clinical study demonstrated limb RIC could reduce the incidence of post-operative atrial fibrillation, acute kidney injury, and it could shorten ITU stay in patients undergoing CABG plus or minus valve surgery, suggesting some benefit on short-term clinical outcomes post-surgery [53]. Crucially, no studies have reported a detrimental effect of the intervention. As mentioned in section 1.4.1, two of the major issues with the previous studies are low numbers and surrogate endpoints. The key to translation for RIC lies in a research collaboration between the UK and Denmark, which will investigate the effect of limb RIC on improving clinical outcomes in STEMI patients treated by PPCI in the RIC-PPCI and CONDI2 trials [126]. With a combined 4400 patients, these studies will give a very good indication as to the potential efficacy of RIC in the setting of PCI. Indeed, these large multi-centre trials are very much game-changers in the cardiovascular field, in that they will change practise if positive and largely kill the field if negative.

### **1.4.3 Summary**

Remote ischaemic conditioning represents a hugely promising cardioprotective intervention, which has not yet had the same impact in clinical studies relative to other animal models of IRI. The primary reason for this, as discussed in section 1.4.1, is a paucity of pre-clinical RIC trials in the literature, meaning there is a lack of basic understanding of the RIC paradigm. If one does not fully understand a physiological phenomenon, it adds great difficulty to potential clinical translation. Clear gaps exist in the current paradigm, which would surely aid design of subsequent clinical trials. This PhD aims to elucidate several key aspects of the communication phase of RIC, focussing on the proposed interaction between the neural and humoral pathways.



## **1.5 The cardiac nervous system**

### **1.5.1 Organisation in the healthy state**

The heart must perform according to the demands of the body at any given moment. This beat-to-beat control of cardiac performance is determined by two efferent inputs to the heart from the central nervous system, working in a reciprocal fashion; the parasympathetic (vagus) nerve is associated with depressing cardiac indices, whereas the sympathetic nerve enhances them. Parasympathetic pre-ganglionic nerves arise mainly from two nuclei in the medulla oblongata, the nucleus ambiguus and dorsal vagal motor nucleus [108]. Efferent parasympathetic axons move to the heart through the vagus nerve and synapse with intrinsic cardiac ganglia. Sympathetic pre-ganglionic nerves arise from the spinal cord, and receive CNS modulation from neurones in the hypothalamus and brainstem [107]. These will synapse with post-ganglionic sympathetic nerves in intrathoracic ganglia, and innervate the sinoatrial and atrioventricular nodes, the atria, ventricles and conducting tissue. Innervation of parasympathetic post-ganglionic nerves within the heart is perhaps more controversial. The original belief, that parasympathetic fibres are sparse or absent from the ventricles, is not supported by the current literature [67]. Rather, many studies have confirmed a wide distribution of vagal postganglionic neurones throughout the ventricles. Projecting from intrinsic cardiac ganglia, they can profoundly influence cardiac rate and rhythm [42].

Sensory innervation to the myocardium is highly complex and originates primarily from afferent neurones with cell bodies in the nodose and dorsal root ganglia [15]. These neurones will transduce the chemical or mechanical milieu within the myocardium and communicate with the central nervous system, which then alters the efferent autonomic tone to the heart. There is, however, an added layer of complexity, as cardiac sensory afferents have also been found in intrathoracic and intrinsic cardiac ganglia, indicating a three-level hierarchical organisation of cardiac neuronal control [16]. Importantly, intrinsic cardiac ganglia contain both parasympathetic and sympathetic post-ganglionic ganglia [14]. Therefore, there exist cardiac

neural reflexes, in the absence of any input from the central nervous system, which can influence cardiac indices; this is known as the intrinsic cardiac nervous system (ICNS).

Several interesting studies have indicated the level of control the ICNS has on cardiac performance. Firstly, an elegant study from McAllen et al demonstrated intrinsic cardiac ganglia were able to gate high frequency input from the CNS [245]. In addition, the synaptic transmission efficiency was not 1:1, indicating these intrinsic ganglia do not act solely as synaptic relay stations for CNS input [11, 245]. Secondly, populations of neurones in both intrinsic and intrathoracic ganglia are able to communicate with each other via local circuit neurones [18]; these are the most common type of nerve found in the heart [13]. Interestingly, local circuit neurones can process afferent and efferent input, influencing both sympathetic and parasympathetic post-ganglionic tone from intrinsic ganglia. This enables the ICNS to control heart rhythm and rate on a beat-to-beat basis [26]. An example of this control is the observation that stabilisation of local circuit neurones, via spinal cord stimulation, reduces the potential for atrial fibrillation in dogs [103]. Thirdly, perhaps the most remarkable trait of the ICNS is its ability to display plasticity. That is, neural networks exist within the heart that can respond to a cardiac event and modify efferent neuronal output for the subsequent few cardiac cycles [15, 181].

The accelerator and brake thesis for cardiac neuronal control is therefore too simplistic. The literature currently suggests a more complex hierarchical cardiac neural control, with sensory feedback loops to central, intrathoracic and intrinsic cardiac ganglia. Local circuit neurones enable efficient processing of the sensory information from the myocardium, and this can alter the efferent autonomic outflow to finely control cardiac performance.

### 1.5.2 The cardiac nervous system and ischaemia-reperfusion injury

Some major innervations of the heart receive their blood supply from non-coronary sources [107]. These presumably would be immune from the deleterious effects of acute myocardial ischaemia, however the effect of infarction of the ICNS is not thought to be due to direct ischaemic injury to the neurones [286]. Sensory afferents within the heart respond to the altered chemical milieu during ischaemia, for example release of purines, free radicals and lactate from dead myocytes [341, 342], leading to reflex changes in sympathetic and parasympathetic tone. The exact influence on efferent output to the heart depends on the region of the myocardium under ischaemia [258], although there tends to be a net tachycardia. Indeed, patients who have a heart rate of >80 beats-per-min following myocardial infarction, indicating high sympathetic tone, have a 39% increased mortality after 1-year [33]. In addition, pharmacological denervation of cardiac sympathetic afferents attenuated some of the deleterious remodelling following heart failure in rats [360].

Thus, the neuronal response to myocardial ischaemia can have a profound effect on the degree of injury post-infarction [190]. Indeed, the complex neuronal hierarchy of the ICNS has not evolved to deal with the extreme sensory environment of myocardial infarction [182], and it can induce structural and functional remodelling of the ICNS [13]. Initial anatomical work revealed that the ischaemic scar contained a much higher density of intrinsic nerves, with an irregular distribution when compared to healthy tissue [353]. This disorganisation, in addition to the increased myocardial noradrenaline post-infarction [172], leads to an increased probability of ventricular and atrial arrhythmias [14, 362].

An elegant study from Rajendran et al. used microelectrode techniques to measure activity of individual intrinsic neurones *in vivo* over the course of myocardial infarction in dogs [286]. It revealed a 'neural sensory border zone', with afferents within the infarct region displaying different properties compared to those within adjacent healthy myocardium. This asymmetry in

afferent sensitivity is thought to underlie the reflex sympatho-activation following infarction [286]. Moreover, these neurones are less sensitive to changes in preload and can no longer communicate as effectively through local circuit neurones.

Finally, recent studies have demonstrated that stimulation of the vagus nerve can ameliorate the post-infarction remodelling of the ICNS [12, 27]. This important finding is discussed further in Chapter 5, where the importance of the vagus nerve and intrinsic cardiac ganglia in acute cardioprotection are investigated.

## **1.6 GENERAL RESEARCH OBJECTIVES**

The overall aim of this PhD project was to investigate the role of the autonomic nervous system in both classical and remote ischaemic preconditioning. This was approached via several experimental projects, the objectives of which are set out below.

**Objective 1. To establish and verify *in vivo* and *ex vivo* models of ischaemia-reperfusion injury, with remote and classical ischaemic preconditioning (IPC) (see Chapter 3)**

Aim 1: Establish and verify a rat Langendorff model of IRI and IPC;

Aim 2: Establish a model of inter-cardiac cardioprotection via coronary effluent transfer;

Aim 3: Establish and verify a model of remote conditioning via transfer of plasma dialysate;

**Objective 2. To perform a systematic review and meta-analysis of animal *in vivo* models of myocardial ischaemia-reperfusion injury (see Chapter 4)**

Aim 1: Ascertain the overall effect and variability of RIC in reducing infarct size in animal models of myocardial ischaemia-reperfusion injury

Aim 2: Investigate the determinants of RIC efficacy, including variables such as RIC protocol and use of supplementary oxygen.

**Objective 3. To investigate the co-dependence of the neural and humoral pathways in remote ischaemic conditioning (see Chapter 5)**

Aim 1: Investigate the role of the vagus nerve in release of the humoral RIC mediator;

Aim 2: Investigate the importance of intrinsic cardiac ganglia in remote ischaemic conditioning.

**Objective 4. To investigate the role of acetylcholine and intrinsic cardiac ganglia in classical ischaemic preconditioning (see Chapter 6)**

Aim 1: Use a pharmacological approach to investigate the role of intrinsic ganglia in myocardial IPC.

**Objective 5. To investigate transcutaneous vagus nerve stimulation as a potential novel cardioprotective intervention (see Chapter 7)**

Aim 1: To measure release of a blood-borne cardioprotective factor following transcutaneous vagus nerve stimulation in human volunteers;

Aim 2: To investigate the cardioprotective potential of transcutaneous vagus nerve stimulation in a rat model of acute myocardial infarction.

## Chapter 2 GENERAL RESEARCH METHODS

Below details the experimental methods employed to investigate the above objectives. More detailed experimental protocols are given in each chapter.

### 2.1 Animal usage

Male Sprague-Dawley rats were used at 250-300g throughout all experiments. All use of animals was in accordance with the United Kingdom (Scientific Procedures) Act of 1986. Rats were housed under 12hr light/dark conditions, with standard chow and water provided *ad libitum*. Animals were bred and cared for by the Biological Services Unit at University College London.

### 2.2 Ex vivo Langendorff perfused heart model of ischaemia reperfusion injury

#### 2.2.1 Preparation of apparatus and perfusion buffer

The perfusion apparatus, as pictured in figure 2-1, was set up as follows. A combination of columns are arranged to facilitate a continuous column of fluid, held at 110cm above a cannula to give a static pressure of ~80mmHg, using the below calculation.

$$p = h \cdot \rho \cdot g$$

Where  $p$  = pressure (Pa),  $h$  = height of the fluid column (m),  $\rho$  = density of the liquid (water is  $\sim 995\text{kg/m}^3$  at  $37.5^\circ\text{C}$ ) and  $g$  = gravitational constant ( $9.81\text{ m/s}^2$ )

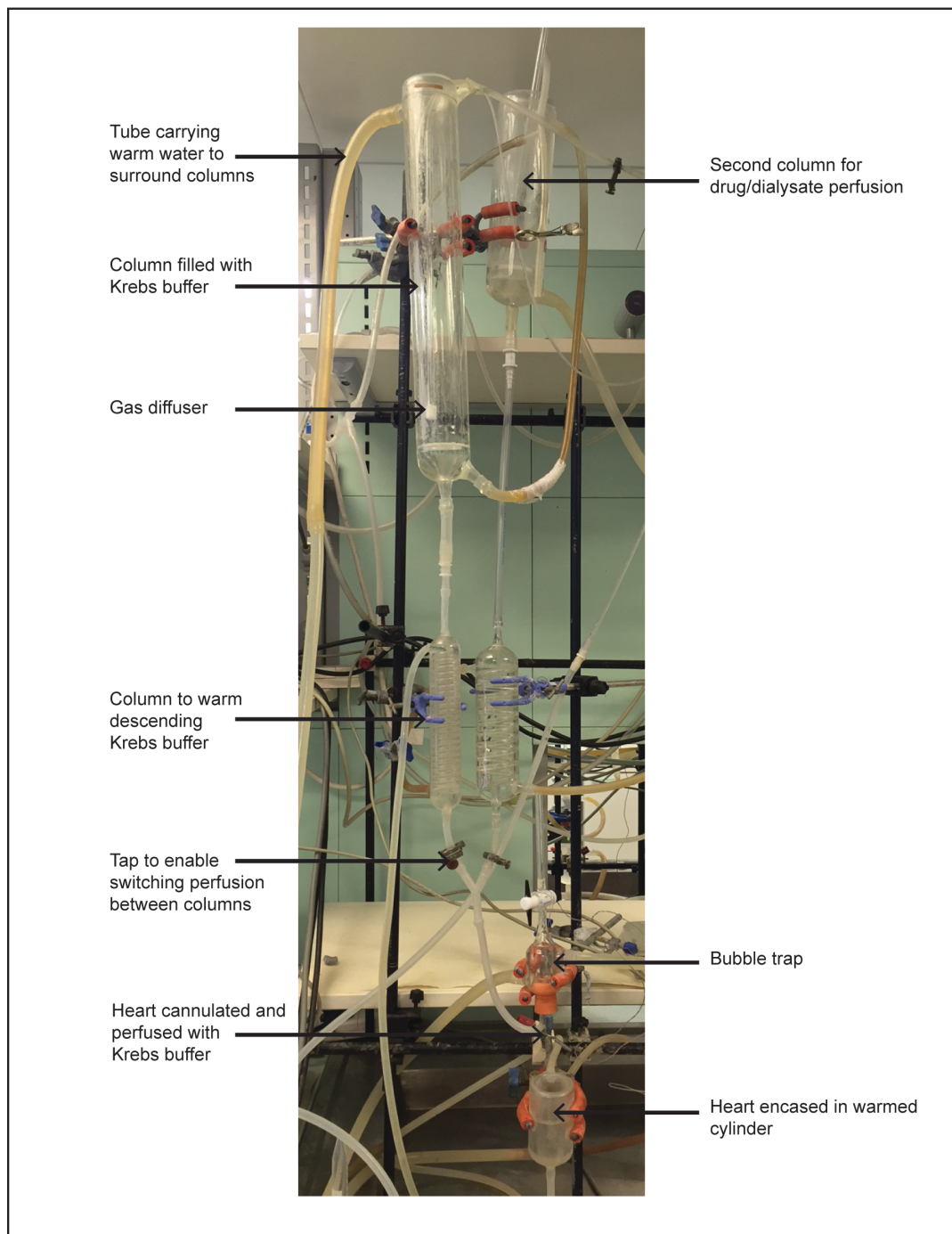
$$\text{Thus } p = 1.10 \cdot 995 \cdot 9.81 = 10.737 \text{ kPa or } 80.3 \text{ mmHg}$$

The columns are filled with a modified Krebs-Henseleit buffer (KHB) consisting of 118mM NaCl, 25mM  $\text{NaHCO}_3$ , 11mM d-glucose, 4.7mM KCl, 1.22mM  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 1.21mM  $\text{KH}_2\text{PO}_4$  and 1.84mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ . The buffer is warmed to  $37.5^\circ\text{C}$  and gassed with 95%  $\text{O}_2$ /5%  $\text{CO}_2$  to obtain a pH of 7.35-7.45.

### **2.2.2 Surgical procedures**

Male Sprague-Dawley rats were anaesthetised with an upper-left quadrant intraperitoneal injection of 60mg/kg sodium pentobarbital (Animalcare, York, UK). Once deep anaesthesia was confirmed by the absence of pedal pain withdrawal reflex, the animal was arranged in a supine position ready for the surgical procedure. A skin incision is made either side of the rib cage at the costal margin to expose the diaphragm and sternum. This incision is continued through the ribs at the left and right anterior auxiliary lines to create a clamshell thoracotomy. The pericardium is then cut-through, exposing the heart and the great vessels, at which point the heart excised via one smooth motion with scissors, ensuring a 5-10mm section of the aorta remains intact. The heart is immediately arrested by placing in ice cold KHB to limit the opportunity for any ischaemic damage before cannulation. The heart is then cleaned of any connective/lung tissue, and the aorta is cannulated with the perfusate already dripping, minimising the risk of air being introduced into the coronary vasculature. The heart is then secured to the cannula with a silk suture. It is worth noting that this cannulation step is absolutely fundamental to the Langendorff method. The cannula must be positioned close enough to the aortic valves so as to create the correct perfusion pressure and enable effective flow through the coronary vasculature. If placed too high, insufficient pressure will be exerted at the level of the coronary ostia, if too low one risks damaging the valves.



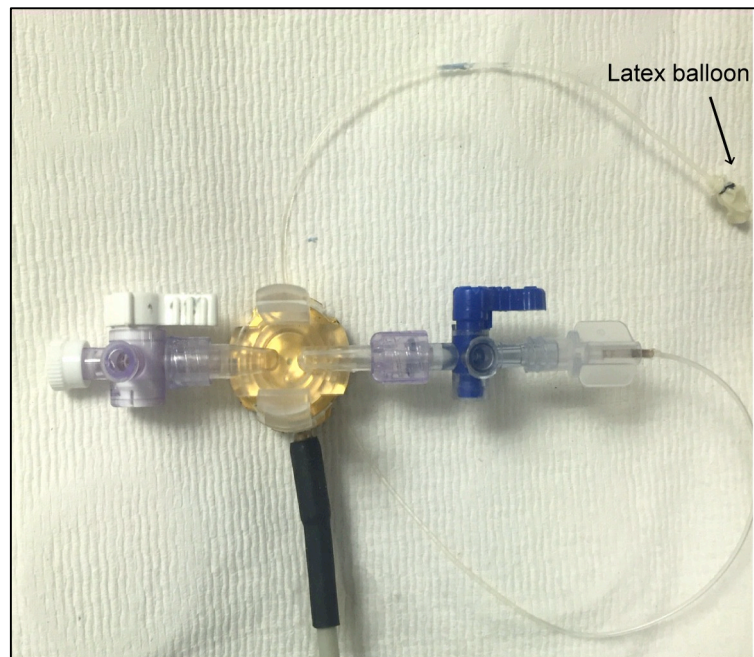


**Figure 2-1. Picture of the Langendorff constant pressure perfusion apparatus used throughout the PhD**

### **2.2.3 Measuring physiological parameters**

The left atrial appendage is removed and a small deflated balloon, made from the tip of an unlubricated latex condom (Durex, UK), is inserted into the left ventricle through the mitral valve. The balloon is attached to a catheter, which connects to a pressure transducer and bridge amplifier (AD Instruments) (figure 2-2). The system is filled with distilled water, which enables pressure changes in the LV to be detected in the pressure transducer. The balloon is inflated to give a left ventricular end diastolic pressure (LVEDP) of 6-10mmHg, providing a physiological preload. The bridge amplifier was connected to a PowerLab/8SP data acquisition system and linked to Chart 7 (both AD Instruments, Oxford, UK) on a computer. One can then visualise the rhythmic pressure of contraction and from it obtain several functional measurements, including LV developed pressure.

A small incision is also made in the right pulmonary opening tract, to facilitate coronary drainage and enable insertion of a temperature probe into the right ventricle. The probe is connected to PowerLab via a T-type pod (AD Instruments, Oxford, UK). Finally, the coronary flow rate can be estimated by measuring the volume of effluent that flows from the heart over the course of a minute. A period of stabilisation is then necessary to allow the heart to become accustomed to its new surroundings and, crucially, to enable the experimenter to determine the quality of the preparation. Several exclusion criteria were referenced to, including coronary flow rate, arrhythmias, heart rate and LV developed pressure, as outlined in table 2-1.



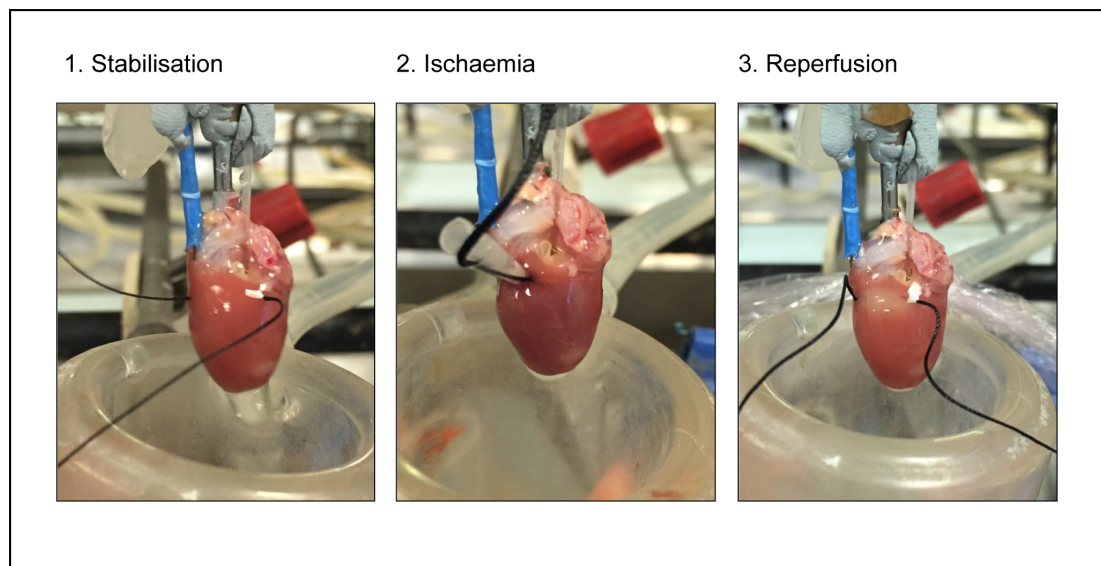
**Figure 2-2 Picture of pressure transducer with latex balloon attached.** The fluid-filled latex balloon would be inserted into the left ventricle of isolated perfused hearts in order to obtain estimates of left ventricular pressures during the cardiac cycle.

Parameter	Criteria for inclusion
Coronary flow rate (mls/min)	>10mls/min, <20 ml/min
Heart Rate (bpm)	>200
LV developed pressure (mmHg)	>70mmHg
Arrhythmias	No continuous arrhythmic episode lasting >3-4 minutes during stabilisation.

**Table 2-1. Criteria to indicate a well-perfused, healthy heart**

#### 2.2.4 Ischaemia-reperfusion injury

Following successful cannulation of the heart, and prior to insertion of the balloon and temperature probe, a 3-0 Mersilk suture (Ethicon, Edinburgh, UK) is inserted through the heart to surround the left anterior descending coronary artery. After a period of stabilisation, the suture is tightened using a snare contraption, which physically closes the left anterior descending coronary artery, thereby inducing ischaemia. This is demonstrated in figure 2-2, where a discolouration of the ischaemic myocardium is clearly visible. To induce reperfusion, the snare contraption is removed, therefore loosening the suture and allowing the LAD to open and reperfuse the ischaemic tissue with buffer. The infarction is clearly visible as a developing pale appearance during the reperfusion period.

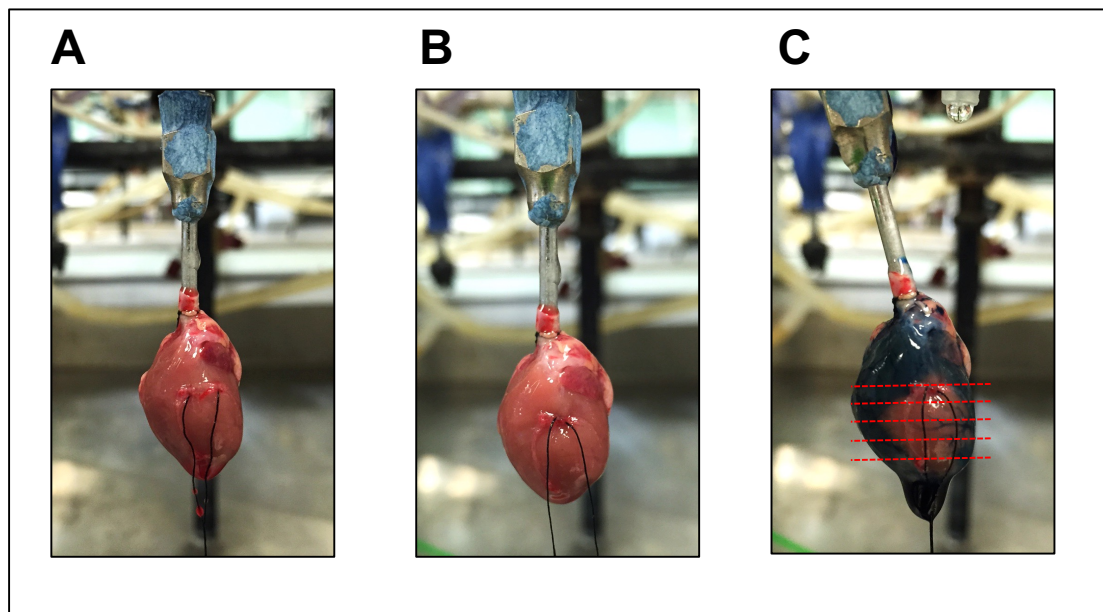


**Figure 2-3. Representative images of an isolated perfused rat heart undergoing ischaemia-reperfusion injury.** A suture is placed around the left anterior descending coronary artery and, after a period of stabilisation, tightened using a snare contraption to induce ischaemia. Reperfusion is initiated by removing the snare contraption.

## 2.2.5 Analysis of infarct size

### 2.2.5.1 Triphenyl tetrazolium chloride staining

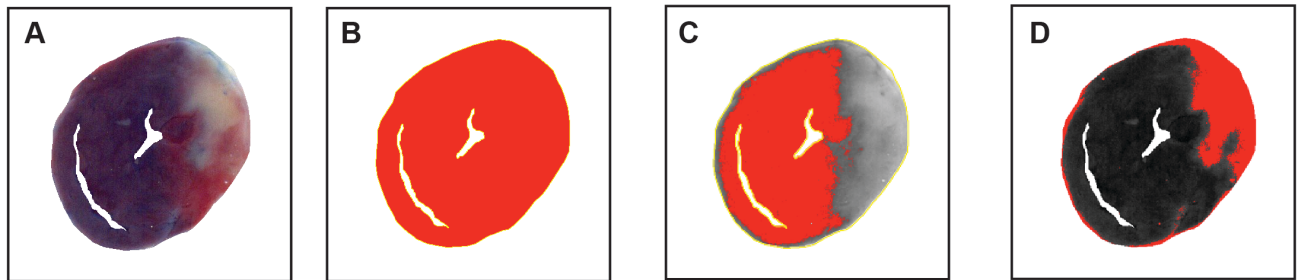
At the end of reperfusion, the suture surrounding the left anterior descending coronary artery was securely re-tied. Around 0.8ml 0.25% Evans blue dye was then injected into the heart through the cannula. This enables visual differentiation between myocardium at risk and not at risk of infarction from the LAD occlusion. That is, myocardium not stained by the dye was at risk of infarction. Samples were then weighed, labelled and frozen for a minimum of 2 hours. The frozen hearts were then sliced into five 2mm sections from the LV suture to the apex, then incubated for 15 minutes in 1% triphenyl tetrazolium chloride (TTC; Sigma-Aldrich, MO, USA) solution (made up in phosphate buffer) at 37.5°C. TTC is reduced by dehydrogenases in live cells to form a red pigment. Non-viable tissue is not stained. Following incubation, the sections are fixed in 10% formalin (Sigma-Aldrich, MO, USA) for at least 2 hours. Heart sections were then scanned to a computer for analysis of infarct size.



**Figure 2-4. Use of Evans blue staining to delineate area-at-risk post-infarction:**

#### **2.2.5.2 Calculation of infarct size using planimetry**

Scanned heart slices were analysed for infarct size using ImageJ software (version 1.45, National Institutes of Health, USA). First, heart sections were isolated and ventricular spaces removed (Fig. 2-5 A). Thresholding was then used to calculate the total ventricular area, the Evans blue area and finally the infarcted area. These three figures could then be used to calculate first the area-at-risk (ventricular area – Evans blue area), and subsequently the infarct size as a proportion of the area-at-risk.



**Figure 2-5. Representative images demonstrating stages of calculating infarct size using ImageJ software.**

*(A) Heart sections isolated and ventricular space removed; thresholding then used to calculate (B) the total heart area, (C) the Evans blue area and (D) the infarcted area.*

## **2.3 Model of cardioprotection via transfer of plasma dialysate**

Below describes a model of cardioprotection used in several publications [164, 331], which enables study of the release of a blood borne cardioprotective factor following an *in vivo* intervention. Particular focus is on remote ischaemic conditioning. This model is employed during chapters 5 and 7.

### **2.3.1 Surgical procedure (rats)**

Male Sprague-Dawley rats (250-350g) were anaesthetised via an upper-left quadrant intraperitoneal injection of 20% w/v sodium pentobarbitone (Animalcare, York, UK) at a dose of 0.05 ml/100g + 0.05ml. Once anaesthesia was reached, confirmed via loss of pedal reflex, the animal was secured in a supine position on a heat mat. A loop of 5-0 suture (Ethicon, Edinburgh, UK) was positioned around the top incisors to enable intubation, via optic light trans-illumination of the trachea, using a modified 16G, 1.7 x 51 mm Abbocath-T intravenous cannula (B. Braun, PA, USA). This cannula was connected to either a PhysioSuite (Kent Scientific, CT, USA) or Small Animal Ventilator (Harvard Apparatus, Kent, UK), and ventilated with air supplemented with 0.5 L/min oxygen. Correct intubation was confirmed via observation of the chest moving up and down with the respiratory cycle. Tidal volumes were calculated using the formula: tidal volume=7.2 ml/kg. A 2cm positive end expiratory pressure was maintained throughout the surgical procedure to prevent collapse of the lungs. Finally, core body temperature was maintained at  $37.0 \pm 0.5^{\circ}\text{C}$  via a rectal probe feeding back to a heat pad. The experiment was terminated if core temperature deviated by more than  $1^{\circ}\text{C}$  either side of this range for more than 5 minutes.

### **2.3.2 Interventions (rat)**

Several different interventions were performed on the anaesthetised rats; these are described in detail during chapters 4,5 and 6. Blood was sampled at the end of each intervention via right ventricular puncture.



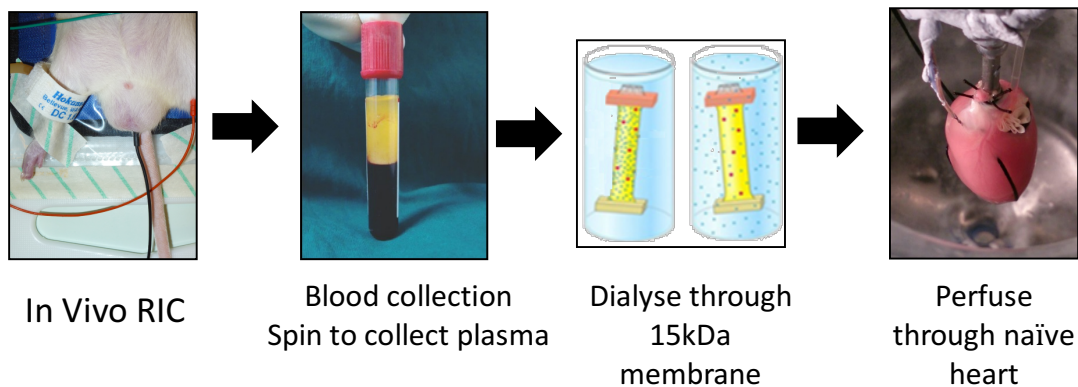
### **2.3.3 Interventions (human)**

As part of a collaboration with the University of Leeds, we performed a study using healthy human volunteers. This is described in detail during chapter 6. Blood samples were taken before and after an intervention via the right antecubital vein.

### **2.3.4 Preparation of dialysate**

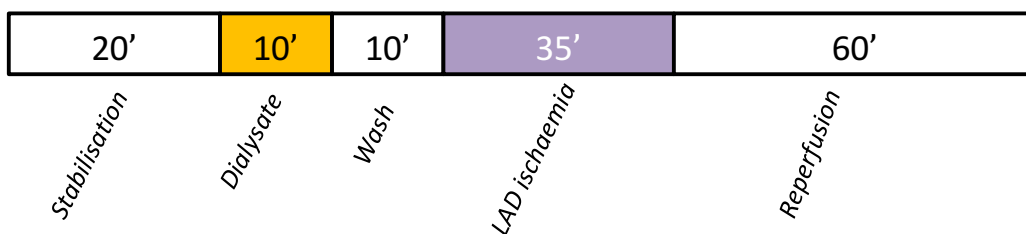
Following blood aspiration, samples were immediately centrifuged at 1600 g for 20 min at 21°C, supernatant collected and spun at 10,000 g for 30 min at 21°C to obtain platelet-free plasma; 9mls of whole blood tended to provide 4 mls of plasma. A 10cm piece of 12-14kDa dialysis membrane (Spectra/Por, Spectrum Laboratories Inc., CA, USA) was soaked for 25 minutes in ddH<sub>2</sub>O, in order to remove the preservative, washing at least twice during this period. This was filled with 4 mls of plasma, clamped at either end, placed in a beaker containing 200 mls of modified KHB (118mM NaCl, 4.7mM KCl, 1.22mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 1.21mM KH<sub>2</sub>PO<sub>4</sub> and 1.84mM CaCl<sub>2</sub>·2H<sub>2</sub>O) and dialysed for 24 hours at 4°C (Figure 2-5). Prior to perfusion through the naïve heart, the dialysate was supplemented with 25mM NaHCO<sub>3</sub> and 11mM d-glucose.





**Figure 2-6: Schematic describing process of dialysate preparation:**

*Animals are randomised to receive either sham procedure or RIC (4 x 5 min hindlimb occlusion and reperfusion). Blood is harvested via puncture of the right ventricle and centrifuged first to isolate plasma and, second, to remove platelets. The plasma is then dialysed across a 12-14kDa membrane before being perfused through a naïve heart prior to IRI.*



**Figure 2-7: IRI protocol for dialysate ex vivo experiments:**

*Isolated perfused rat hearts were, after a 20-minute stabilisation period, perfused with either control or RIC dialysate for 10 minutes. After a 10 minute washout period, the hearts were put into ischaemia via LAD occlusion for 35 minutes, followed by 60 minutes of reperfusion.*

### **2.3.5 Ex vivo ischaemia-reperfusion injury**

Sprague-Dawley rats (250-350g) were anaesthetised with an upper-left quadrant intraperitoneal injection of 60mg/kg sodium pentobarbital (Animalcare, York, UK). Following confirmation of deep anaesthesia, a clamshell thoracotomy was performed, the heart excised and immediately placed in ice cold KHB. The aorta was then cannulated and coronary vasculature perfused with KHB using a Langendorff gravity driven constant pressure rig as previously described (section 2.2).

The hearts then underwent a 20 minute period of stabilisation, followed by perfusion of the dialysate for 10 minutes and a subsequent 10-minute washout period prior to a 35 minute left anterior descending coronary artery (LAD) occlusion and 60 minute reperfusion (see Figure 2-7). At the end of reperfusion, infarct size was evaluated using methods described in section 2.2.5.

### **2.3.6 Blinding and data analysis**

The experimenter was not blinded to either application of RIC or sham protocols to the anaesthetised rat, nor the subsequent dialysate perfusion through the naïve isolated rat hearts. The experimenter was, however, blinded to the analysis of infarct size for each heart. Data analysis was conducted using Prism; detailed analysis protocols are provided in each chapter.

## **2.4 Ex vivo model of isolated primary cardiomyocyte hypoxia-reoxygenation**

Male Sprague-Dawley rats were anaesthetised with 60mg/kg sodium pentobarbital, hearts excised and cannulated on a modified constant-flow Langendorff apparatus. The hearts were then perfused with a series of five solutions, based on an isolation buffer described in table 2-2. Solutions were warmed to 37.5°C and gassed with a 100% oxygen. *Solution 1*, containing a small amount of calcium, was perfused through the heart for 4 minutes to clear the heart of blood and rejuvenate the heart following cannulation.

*Solution 2* contained 100 $\mu$ M EGTA and was perfused for 4 minutes in order to arrest the heart and prevent myocyte hypercontracture. *Solution 3* contained 100 $\mu$ M CaCl<sub>2</sub> and 0.5mg/ml type II collagenase (Worthington, NJ, USA) and was perfused through the heart for 8 minutes. The heart should appear pale and swollen. At the end of this stage, the ventricular tissue is cut from the heart and then cut into small pieces using scissors with the tissue bathed in 10mls of solution 3. This solution is then gassed with oxygen and continuously agitated. Here, the experience of the operator plays a role, as one must observe the right moment to stop the digestion. Too little time, and the yield of cells is too small, too long and the collagenase will induce myocyte death. The cells are allowed to pellet and calcium slowly re-introduced via *solution 4* (0.5mM CaCl<sub>2</sub>) and *solution 5* (1M CaCl<sub>2</sub>). Myocytes were finally resuspended in M199 medium with added penicillin (100 IU/ml), streptomycin (100 IU/ml), L-carnitine (2 mM), creatine (5 mM), taurine (5 mM) and BSA (2g/L) (Medium 199, Sigma, UK), before plating on 35mm dishes (VWR international, PA, USA) coated with laminin and left to stabilise 24hr prior to commencing the experiment.

Salts	Molarity	g/500ml
NaCl	130	3.79
KCl	5.4	0.2
MgCl <sub>2</sub>	1.4	0.066
Na <sub>2</sub> HPO <sub>4</sub>	0.4	0.028
HEPES	4.2	0.5
<b>pH to 7.3 at 37°C</b>		
Glucose †	10	0.9
Taurine †	20	1.25
Creatine †	10	0.655

**Table 2-2: Stock perfusion buffer components for cardiomyocyte isolation**

Solution number	Additional components	Required addition to volume stated in first column
(1) 50ml; to clear the heart of blood	750 $\mu$ M CaCl <sub>2</sub>	37.5 $\mu$ l of 1M CaCl <sub>2</sub> stock solution
(2) 50ml; to arrest the heart	100 $\mu$ M EGTA	2mg of EGTA
(3) 40ml; to digest the heart	100 $\mu$ M CaCl <sub>2</sub> 0.5mg/ml Collagenase	40 $\mu$ l of 100mM CaCl <sub>2</sub> 15-20mg Collagenase
(4) 50ml; to reintroduce calcium	0.5mM CaCl <sub>2</sub>	25 $\mu$ l of 1M CaCl <sub>2</sub>
(5) 50ml; to reintroduce calcium	1mM CaCl <sub>2</sub>	50 $\mu$ l of 1M CaCl <sub>2</sub>

**Table 2-3. Additional components to each solution to be prepared fresh prior to the isolation procedure**

## **2.5 Biochemical assays**

### **2.5.1 Bicinchoninic acid (BCA) assay to measure protein content**

Plasma or dialysate samples were assayed for protein concentration using the BCA assay method as described previously [327]. Briefly, 5µl of each sample is added to a 96-well plate, followed by 195µl of a 1:50 mix of copper II sulphate and bicinconinic acid. This is allowed to incubate for 30mins at 37°C before being subjected to colorimetric analysis at 470nm using an automated 96-well plate reader (Fluostar Omega, BMG Labtech, UK).

The assay is dependent on two reactions. First, a temperature dependent reaction where peptide bonds in the sample reduce  $\text{Cu}^{2+}$  to  $\text{Cu}^{+}$ . The amount of  $\text{Cu}^{2+}$  reduced is proportional to the protein content of the sample. Second, bicinchoninic acid chelates with  $\text{Cu}^{+}$  to form the distinctive purple colour associated with the assay. This can then be read using the automated plate reader described above.

### **2.5.2 Acetylcholine assay**

A Choline/Acetylcholine Assay Kit (Abcam, UK) was used to measure the concentration of acetylcholine in effluent collected following IPC (3x5min global ischaemia-reperfusion) or corresponding control period, as described above. The assay was carried out in accordance with the instructions provided by the manufacturer. Briefly, via the use of acetylcholinesterase, the level of free and total choline was measured in each sample, enabling an estimation of the concentration of ACh within the sample.

## Chapter 3 CHARACTERISATION OF THREE MODELS OF CARDIOPROTECTION

### 3.1 Introduction

Soon after the discovery of IPC, research from the Przyklenk group gave the first clue to the remote potential of the intervention [284, 371]. In an *in vivo* rat model of IRI, they found that infarct size of preconditioned hearts, as a proportion of the area at risk (I/AR), was positively correlated with the area at risk, as a proportion of the left ventricle (AR/LV). That is, the larger the AR/LV, the larger the IS/AR. No correlation was observed with control hearts. This, they suggested, meant the non-ischaemic region contributed to the cardioprotection afforded by IPC. Indeed, this observation was corroborated in pigs [113], and a mathematical model of this phenomenon suggested a factor could diffuse from the non-ischaemic region to the AR and add to the protection [371]. Although this hypothesis of diffusion has proved incorrect, the experiment that precipitated from this correlation was the first observation of RIC, and initiated a huge research field.

Further to the 'preconditioning at a distance' first described by Przyklenk et al [283], several other groups extended the paradigm demonstrating application of a conditioning stimulus to the kidneys, mesentery or gastrocnemius muscle could induce cardioprotection [35, 102]. These data necessitated a communication step, and it was suggested a humoral factor was released in response to conditioning that moves to the heart via the bloodstream to induce protection. Przyklenk's group followed up this hypothesis with an elegant study [75]; coronary effluent collected from an isolated perfused rat heart subjected to IPC could induce cardioprotection when perfused through a naïve isolated heart prior to IRI. Thus, the presence and necessity of a blood-borne cardioprotective factor was clear. It is worth noting, however, that the Downey group was not able to replicate the original 'PC at a distance' experiment in a rabbit model [257].

Whilst this model of inter-cardiac conditioning has proved a useful experimental tool, as the paradigm of RIC expanded it became clear the

mechanism of communication was more complex than a humoral mediator released from the conditioned limb. Indeed, several experimental studies demonstrated the dependence of RIC on intact neural innervation to the conditioned limb [102, 222, 223]. However, these models were *in vivo* in nature, meaning one could not ascertain exactly what role the neural pathway plays in the RIC paradigm. Thus, a new model of RIC was required, containing both *in vivo* and *ex vivo* components, which would enable differentiation between the trigger, communicator and effector components. Developed by the Redington laboratory, it involved preparation of a plasma dialysate following *in vivo* RIC in rabbits, which was then perfused through a naïve heart prior to IRI [320]. The model therefore investigates the release of a humoral factor into the blood following RIC and, crucially, enabled the experimenter to intervene at the *in vivo* stage to test the regulation of release of the humoral mediator. Several publications from groups using this model have demonstrated the dependence of the humoral pathway on activation of sensory afferent nerves from the conditioned limb [164, 288, 331]. This was a huge paradigm shift, highlighting the complexity of the phenomenon.

Via use of both the models described above, this thesis aims to expand the RIC paradigm further, with particular emphasis on the neural pathway in both the communication of RIC and the induction of protection within the myocardium [278].

### **3.2 Research aims and objectives**

The primary objectives of the chapter were three-fold:

1. To establish and validate a model of ischaemia-reperfusion injury using the Langendorff isolated perfused heart method;
2. To use the above model to recreate the inter-organ model of classic IPC via coronary effluent transfer.
3. To recreate and validate the model of RIC using transfer of plasma dialysate.

### **3.3 Aim 1: Establish *ex vivo* model of myocardial ischaemia-reperfusion injury**

#### **3.3.1 Background**

Oskar Langendorff first proposed the concept of retrograde perfusion of an isolated mammalian heart in 1895 [208], based on earlier work by Elias Cyon on the isolated frog heart. Indeed, the basic principles have largely remained the same since and the technique has proved very useful in understanding cardiac physiology. In particular, this model has played a central role in our understanding of cardioprotection, from initial discovery of ischaemic preconditioning [255] to the identification of protective signalling pathways [129]. The simplicity and reproducibility of the model, along with the ability to study the heart in isolation of other systemic influence, are its primary advantages.

The isolated heart, following excision, is retrogradely perfused with a crystalloid buffer via cannulation of the aorta. The pressure created via the perfusate will close the aortic valve and force the buffer into the coronary arteries via the ostia. The perfusate will then pass through the vascular bed of the heart and drain as normal into the right atrium via the coronary sinus. In the absence of the pulmonary system, the buffer is then free to drain from the heart, thus the heart beats away happily without any fluid filling of the ventricles.

The original method of perfusion, first created by Langendorff, is via maintenance of a constant fluid pressure at the aortic root of the isolated heart. This was achieved via a sealed pressurised chamber and monitored by an attached manometer [208]. Modern, commercially available, apparatus is designed in a similar style, however one maintains constant perfusion pressure via a negative feedback peristaltic pump. The advantage here is that a small volume of fluid is needed to create the necessary pressure for perfusion. This enables use of blood supplemented perfusion buffers, or investigation of a drug with limited availability or high expense. In addition, one is able to measure coronary flow rate continuously and can switch



seamlessly to a constant flow set-up. With a known flow rate and perfusion pressure, a derivation of Ohm's law can be used to calculate coronary vascular resistance. This mode is therefore preferred for investigating coronary vascular tone, smooth muscle or endothelial function. Conversely, the constant pressure mode has been well validated in the setting of acute ischaemia-reperfusion injury.

The disadvantage of the commercially available Langendorff apparatus, however, is the peristaltic pump. This, in the author's experience, is very prone to dysfunction and unnecessarily complicates the maintenance of constant perfusion pressure. Indeed, this can be easily induced via a single column of fluid, with the meniscus set at a known distance above the cannula tip. Once the aorta of the heart is cannulated, the static fluid column will maintain the pressure at the aortic root, and the coronary circulation can naturally choose the rate of flow. This is the mode of perfusion used throughout this thesis.

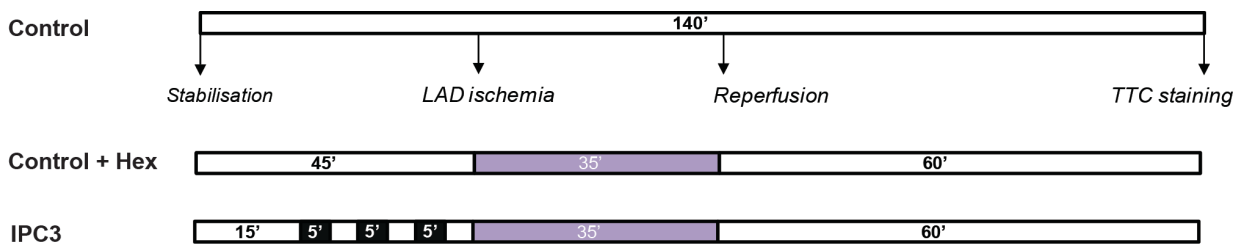
The Langendorff model has been used extensively in the setting of acute ischaemia-reperfusion injury. This is easily induced via tightening/loosening of a silk suture placed around a coronary artery, and then quantifying the cell death that occurs. The model can therefore quickly give an idea as to the cardioprotective potential of a particular drug or intervention.

The aim for this section was to establish and validate a model of IRI using isolated perfused rat hearts. To do this, it is necessary to demonstrate a reproducible infarct size in response to an injury, with a standard deviation small enough to detect a cardioprotective intervention. Here, IPC will be used as a positive control.

### 3.3.2 Methods

The methods used are described in detail in section 2.2. Isolated perfused rat hearts were randomised to receive one of three protocols: (1) sham procedure, where the heart is perfused without intervention for 140min; (2) Control, where hearts received 45min stabilisation followed by 35min left anterior descending artery ischaemia and 60min reperfusion; (3) IPC, hearts received 3 cycles of 5min global ischaemia-reperfusion immediately prior to index ischaemia as described above.

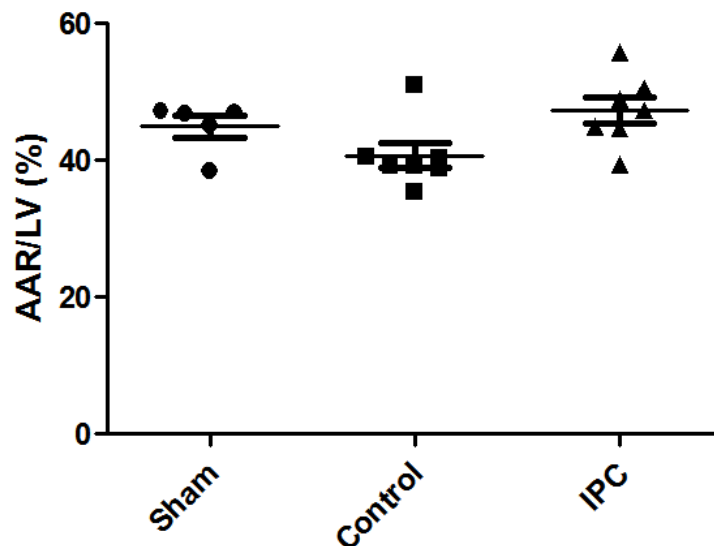
Following each experiment, hearts were analysed for infarct size using triphenyl tetrazolium chloride staining (described in section 2.2.5). An indication of functional recovery from ischaemia was observed via measurement of left ventricular developed pressure and coronary flow rate. In addition, both coronary flow rate and left ventricular developed pressure were measured at specific time points throughout the experimental protocol (described in section 2.2.3).



**Figure 3-1. Experimental protocols during characterisation of the Langendorff isolated heart model.**

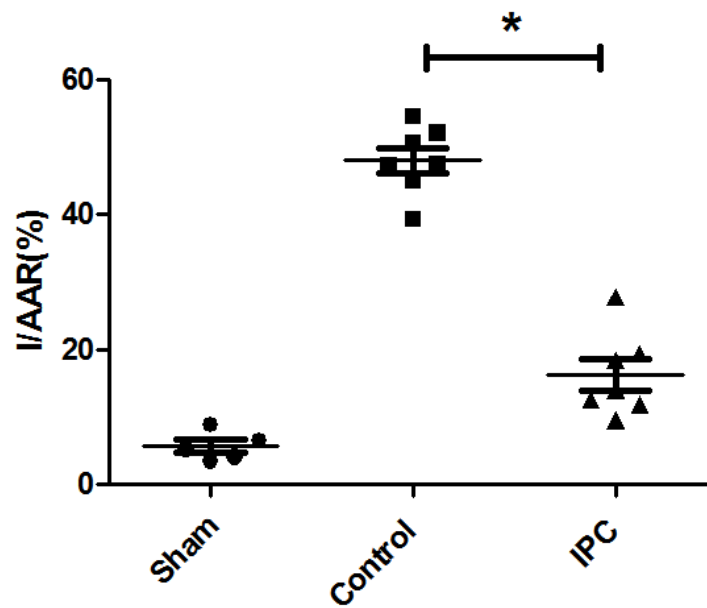
### 3.3.3 Results

An important step in validation of the model is ensuring reproducibility of suture placement. Indeed, the area at risk needed to be between 35%-65% of the LV area for inclusion in the final data set. If one can maintain a constant AAR, then any variability in infarct size is reduced. Too large an AAR would induce a large injury from which the heart may not recover, too small would induce a smaller injury from which the heart would recovery well. Figure 3-2 shows the AAR% in each of the groups, with no significant differences between the groups thus indicating procedural consistency.



**Figure 3-2: Area at risk in isolated rat hearts subjected to either sham, control or IPC protocols.**

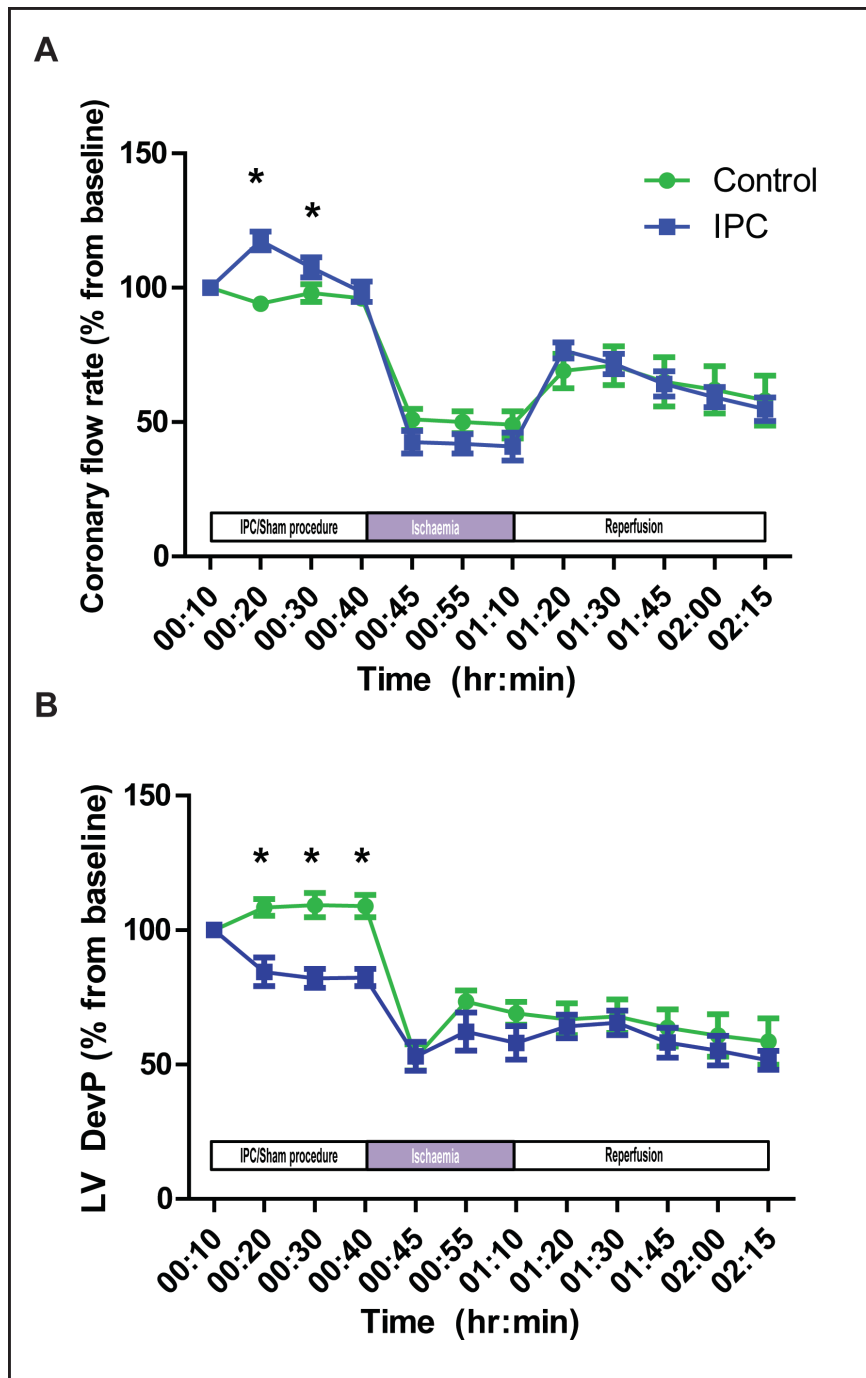
*No statistical difference was found between the groups, thus confirming surgical consistency in placement of LAD suture.*



**Figure 3-3: Comparison of infarct size between sham, control and IPC groups.**

*The ischaemia-reperfusion protocol induced a reproducible injury in isolated rat hearts. IPC was able to significantly reduce infarct size relative to control ( $40.7 \pm 4.4\%$  vs  $23.0 \pm 4.8\%$ ,  $p < 0.05$ ). Normality confirmed via Kolmogorov-Smirnov test. Statistical significance was assessed using one-way ANOVA with Tukey's multiple comparison test.*

Infarct size analysis revealed that the IR protocol induced considerable and reproducible injury to the myocardium ( $I/AAR = 48.0 \pm 1.9\%$ ). This injury was significantly attenuated by the preceding IPC protocol ( $I/AAR = 16.2 \pm 2.4\%$ ,  $p < 0.05$  vs control). This level of cardioprotection is very much in line with the literature [158, 227, 250, 354], thus further validating the model characterisation. Sham hearts displayed infarcts of  $5.7 \pm 1.0\%$ , confirming the procedure of hanging and retrogradely perfusing the heart is associated with minimal myocardial damage.



**Figure 3-4. Functional parameters measured in hearts who received either sham or IPC.**

Measurement of (A) coronary flow rate and (B) left ventricular developed pressure, in naïve hearts that received control or IPC procedures, throughout the ischaemia-reperfusion protocol. All data presented as mean  $\pm$  SEM, data analysed by two-way ANOVA.

Finally, no significant improvement in functional recovery from index ischaemia was observed with IPC, both in terms of coronary flow rate and left ventricular developed pressure (Fig. 3-4). Interestingly, the coronary flow rate increased significantly during the IPC protocol, particularly after the first cycle. In addition, during the IPC protocol the developed pressure recovered from each ischaemic cycle to only 80% of baseline.

### 3.3.4 Discussion

The Langendorff model of ischaemia-reperfusion injury has been extensively described in the literature, and is very well established in the literature. Thus, although there are several key caveats to the model, this project did not revisit them in the interests of time. It is important, however, to discuss these caveats, which include species, gender, age, use of anaesthetic and duration of IR.

- *Species.* The choice of species is key to the Langendorff IRI model [368], due to the inherent variability in the coronary vasculature [244]. Small animals such as rabbits, rats or mice are traditionally used due to their small size, thus smaller apparatus and less perfusate are required, in addition to the reduced cost per animal. Despite the obvious limitations to using small mammalian hearts, notably the faster heart rate, altered  $\text{Ca}^{2+}$  handling and limited collateral flow, they are suitable to the buffer perfused Langendorff model as a homogenous supply of the myocardium with oxygen and nutrients is easier to ensure. The advantage of the Langendorff model, as previously mentioned, is its simplicity; the use of small animals facilitates this simplicity.
- *Gender.* Male rats are almost exclusively used in the study of myocardial IRI. This is due to the potential confounding oestrus cycle in females. Indeed, oestrogens are associated with significant cardio-atheroprotection, highlighted perhaps by the reduced CAD incidence in females [252], and reviewed nicely by Hodgkin and Maeda [146]. There are clear gender differences in the efficacy of cardioprotection

[21], which have not been fully defined. Therefore, although IRI is very much pertinent to both sexes, only male rats were used in this thesis to avoid an unnecessary confounder.

- *Age.* The rats used in the model were between 3 and 5 months of age, which correlates with a young adult stage of life. Age has been well documented to influence the cardiac response to IRI and cardioprotective interventions [372]. The effect of age on the cardiovascular system is very well described, which precipitates as increased incidence of CAD. Thus, investigating IRI in young rats is perhaps not the ideal representation of CAD in humans, most commonly developing during middle/old age.
- *Duration of IR.* The standard IR protocol used in this laboratory for isolated hearts is 35 minutes LAD occlusion followed by 60 minutes reperfusion. This protocol induces an infarct size that enables effective demonstration of cardioprotective interventions such as IPC and RIC. The extent of infarction is very much correlated with the duration of ischaemia. Whilst reperfusion duration has been recently demonstrated to correlate with infarct size in mouse Langendorff [301], in the rat model 60min reperfusion gives a similar infarct size to 120min [93]. Thus, 60min reperfusion was used throughout this thesis. This protocol was very tightly controlled for each experiment.
- *Temperature and pH.* These are the two most important variables to maintain at a consistent level in the Langendorff model [29]. Indeed, van Vuuren et al. demonstrated that a very small (0.5°C) reduction in temperature impacted the ability to cardioprotect with IPC [355]. In addition, pH has a great influence not only on normal cardiac function but also the response to ischaemia reperfusion injury.

The use of functional recovery as a valid endpoint in the Langendorff model is controversial. The nature of the model means that no fluid enters the left ventricle, instead preload is created artificially via insertion of a balloon (section 2.2.3). Although the extent of infarction is associated with myocardial function [228], several studies have failed to see a recovery of function proportional to the reduction in infarct size induced with IPC [64, 161, 345].

Therefore, infarct size is seen as the more appropriate end point in studies investigating cardioprotective interventions. This will be used as the primary endpoint throughout this thesis.

### **3.4 Aim 2: Establish model of inter-cardiac cardioprotection via transfer of coronary effluent**

#### **3.4.1 Background**

The first demonstration of cardioprotection via transfer of coronary effluent came from the Przyklenk group in 1999 [75]. Coronary effluent collected from an isolated rabbit heart during an ischaemic preconditioning protocol could induce powerful cardioprotection when perfused through a naïve isolated rabbit heart prior to IRI. Effluent collected during a time-matched control period offered no protection to the naïve acceptor heart. This provided the conclusive evidence for a humoral factor as the communicative agent for IPC. Crucially, it provided the opportunity to intervene at the level of the effluent and directly investigate the identity and source of the humoral factor. For example, adding an antagonist to the effluent, or analysing for presence of a particular molecule/protein.

Given the similarity of this model to the initial demonstration of remote ischaemic conditioning [283], this was interpreted as proof of a humoral mediator for this paradigm. However, this is likely not the case as RIC is communicated via a complex neuro-humoral mechanism involving, for example, the dorsal vagal nucleus in the brainstem. Thus, the model demonstrates that IPC is triggered and mediated via release of several factors, which locally induce protection.

Several important additions to the IPC paradigm have been made using the effluent model. Given IPC was thought to be receptor-mediated, via small molecules such as adenosine and opioids, studies have focussed on these as potential mediators of IPC, with contrasting results. Initially, Dickson et al could not find any correlation between infarct size and adenosine concentration in the IPC effluent [75]. Instead, a further study from the same



group suggested opioid involvement, with naloxone abrogating IPC-effluent mediated cardioprotection [74]. However, pre-treatment with exogenous Met- and Leu-enkephalin failed to elicit a cardioprotective response. In addition, contrasting evidence from the Redington group implicated adenosine as a mediator of effluent-mediated protection; adenosine was significantly increased in IPC-effluent and receptor blockade with 8-(p-sulfophenyl)theophylline (8-SPT) abrogated IPC-effluent cardioprotection [215]. Thus, the combined data suggests the possibility of opioid and adenosine receptor crosstalk, a phenomenon implicated in both IPC [266] and RIC [335].

The second important contribution the effluent model provided was a characterisation of the protective factor, with the current paradigm suggesting that it is a hydrophobic, thermolabile protein between 3.5kDa and 30kDa in weight [138, 215, 317]. This would suggest small molecules (such as opioids and adenosine) are not essential for protection. Indeed, there is likely some redundancy in this communication step, with more than one cardioprotective factor released.

Thirdly, the model has been used to gain important insights into the mechanism of induction of protection in the myocardium. Traditional cardioprotective kinases were implicated, including activation of PKC, ERK, Akt and PI3K. In addition, two studies have demonstrated that preservation of mitochondrial integrity is fundamental to effluent-mediated protection, via inhibition of ROS production, preserved outer membrane integrity, improved respiration and inhibition of mPTP formation [215, 346].

The aim of this section was to recreate the model of inter-cardiac conditioning using isolated rat hearts, to enable investigation of the humoral communication of IPC. In addition, an exploratory experiment was undertaken to investigate the involvement of stromal-derived factor 1 alpha in this setting. This is a highly cardioprotective chemokine, acting through the CXCR4 receptor, and has recently been implicated in the mechanism of remote ischaemic conditioning [390].

### 3.4.2 Methods

#### 1) *Model characterisation*

Isolated perfused rat hearts were used throughout this experiment. Detailed methodology for this model is found in section 2.2. Hearts were randomised firstly to be either donors or acceptors, and subsequently to either control or IPC groups, thus four groups in total : (1) *Donor Control* hearts underwent 30 minutes of perfusion during which effluent was collected; (2) *Donor IPC* hearts received three cycles of 5-minute global ischaemia with intermittent 5-minute reperfusion, during which effluent was collected; (3) *Acceptor Control* hearts received 10 minute perfusion of donor control effluent immediately prior to ischaemia; (4) *Acceptor IPC* hearts received a matched 10 minute perfusion of donor IPC effluent immediately before ischaemia. In all groups, hearts were subjected to 35 minutes of LAD ischaemia and 60 minute reperfusion.

Three experimental endpoints were used. First, infarct size was measured in each heart at the end of reperfusion, using Evan's blue to delineate the area at risk and triphenyl tetrazolium chloride staining to measure cell death (section 2.2.5).

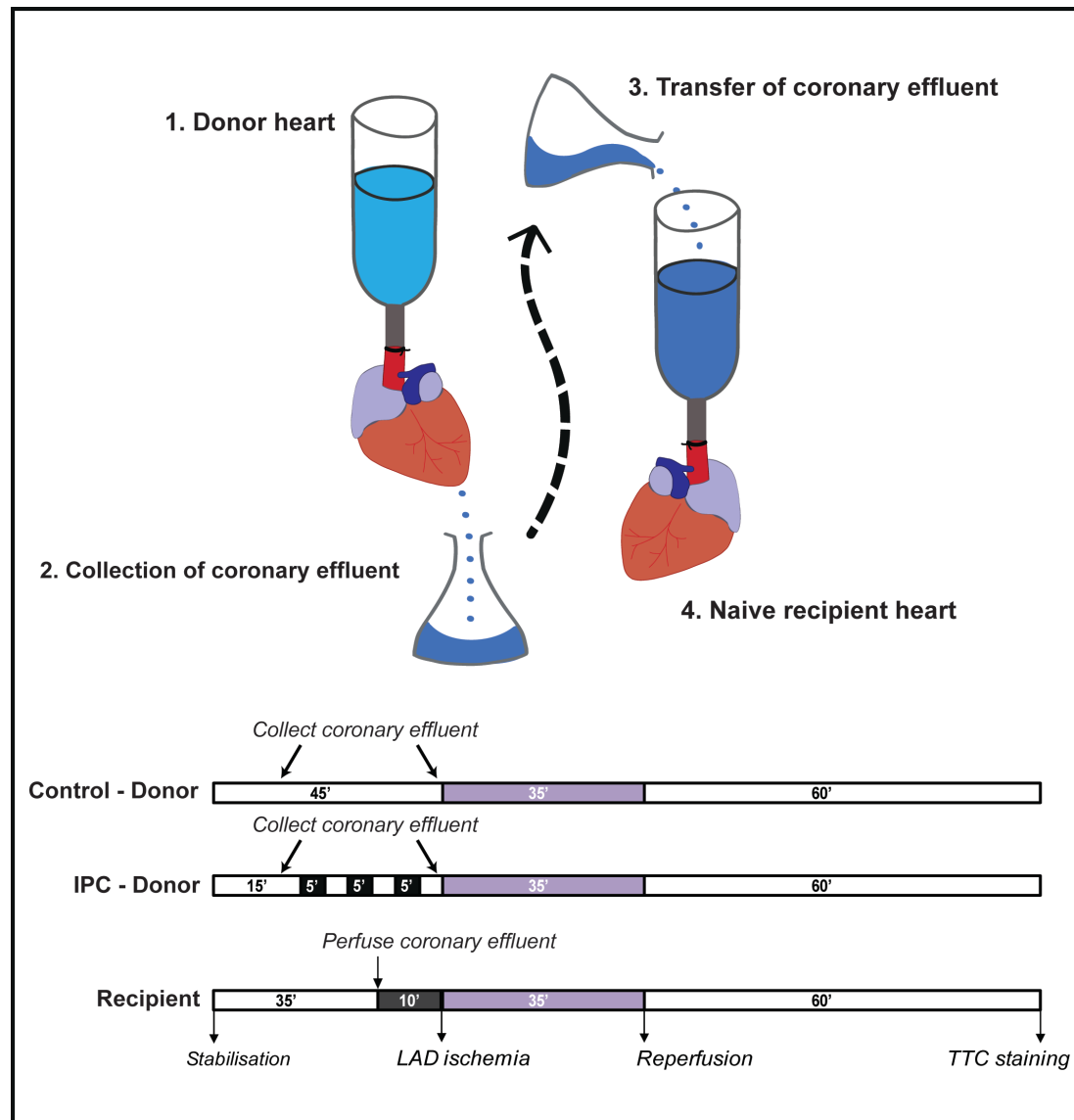
Second, several functional parameters were taken from the perfused heart throughout the IRI protocol. Pressure and heart rate measurements were taken via a fluid-filled balloon in the left ventricle. In addition, coronary flow rate was manually measured at set time points throughout the protocol. These are described in more detail in section 2.2.3.

Finally, protein concentration in both control and IPC effluent was measured using the BCA method described in section 2.6.

## *2) Study to investigate importance of stromal-derived factor 1 alpha*

Stromal derived factor 1 alpha (SDF-1 $\alpha$ ) has powerful cardioprotective properties through its activation of CXCR4, and recently has been implicated in the mechanism of remote ischaemic conditioning [72, 390]. We therefore wanted to test whether it was important in this setting. Again, effluent was collected from donor hearts following either IPC (3 x 5 min global ischaemia-reperfusion) or a corresponding control period. This was perfused through a naïve isolated heart prior to index ischaemia as described above. The CXCR4 antagonist, AMD3100, which prevents SDF-1 $\alpha$  binding, was perfused for 5min prior to and the duration of effluent perfusion (Figure 3-9).

Two experimental endpoints were used. First, Infarct size was calculated at the end of reperfusion as described in section 2.2.5. Second, coronary flow and left ventricular developed pressure were measured at set time points during the IRI protocol to give an indication of heart function.



**Figure 3-5. Schema of the effluent transfer model and experimental protocols.**

*Effluent was collected from donor hearts following either IPC or corresponding sham protocols. This effluent was subsequently perfused through a naïve isolated rat heart immediately prior to a 35min left anterior descending coronary artery ischaemia and 60min reperfusion. Donor hearts also received the same ischaemia-reperfusion injury protocol following effluent collection. Hearts were analysed for infarct size at the end of each experiment via TTC staining.*

### 3.4.3 Results

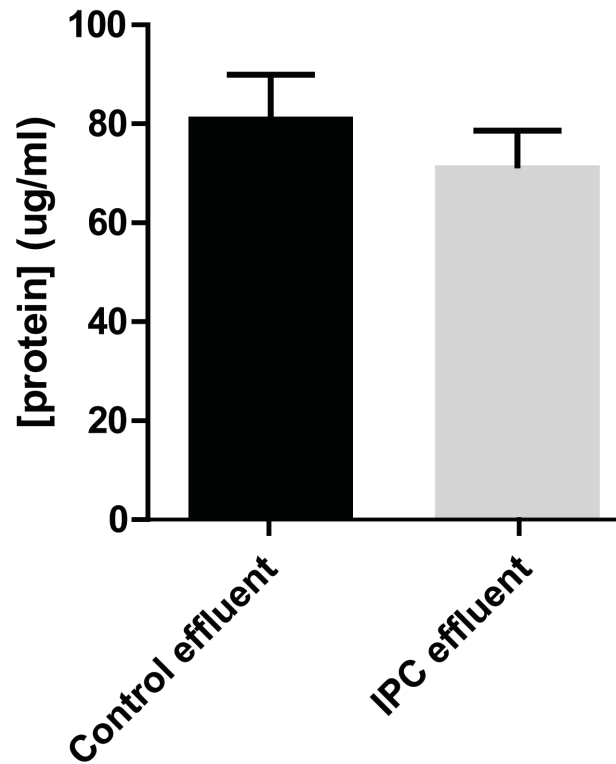
#### *Coronary effluent protects naïve isolated rat hearts from IRI*

There was no difference in observed protein concentration between control and IPC effluents (IPC =  $72 \pm 7$   $\mu\text{g/ml}$  vs Control =  $81 \pm 9$   $\mu\text{g/ml}$ ,  $p = 0.43$ ) (Figure 3-4).

Infarct analysis revealed powerful cardioprotection to those hearts treated with IPC effluent (IPC effluent I/AAR % =  $17 \pm 3$  vs control effluent =  $42 \pm 2$ ,  $p < 0.05$ ) similar to that observed with direct IPC ((Donor IPC I/AAR % =  $22 \pm 6$  vs donor control =  $41 \pm 5$ ,  $p < 0.05$ ) (Figure 3-7). Thus, IPC effluent seems to contain within it a cardioprotective protein or molecule that offers significant protection to the acceptor heart.

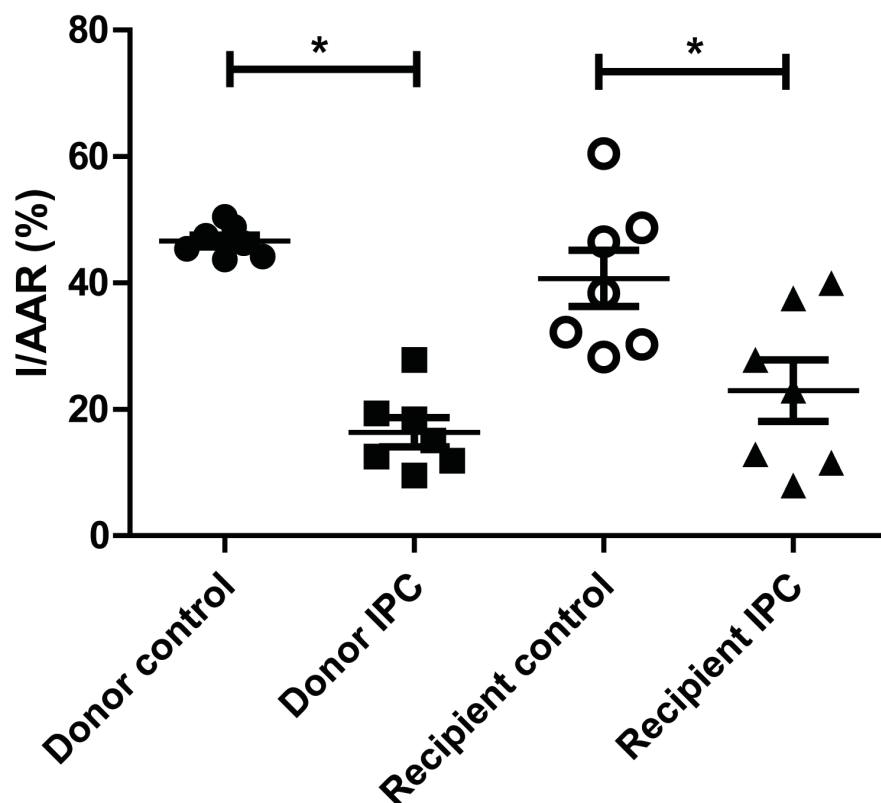
Functional parameters are expressed as coronary flow rate and left ventricular developed pressure, measured throughout the experimental protocol (figure 3-7). There was a small, albeit statistically non-significant, recovery of function following ischaemia in both endpoints. Interestingly, a significant increase in coronary flow was observed following IPC effluent perfusion, indicating a vasodilation in the naïve heart and the presence of a haemodynamic factor in the effluent.

There was a clear, but non-significant, correlation between the extent of vasodilation and infarct size ( $r^2 = 0.63$ ,  $p = 0.06$ ,  $n = 6$ ), suggesting that the vasodilator may also contribute to cardioprotection (Fig. 3-8). In addition, there seemed to be a positive correlation between infarct size of donor and acceptor IPC hearts ( $r^2 = 0.48$ ,  $p = 0.09$ ,  $n = 7$ ), not existent for control ( $r^2 = 0.21$ ,  $p = 0.36$ ,  $n = 6$ ), although this correlation was again not significant.



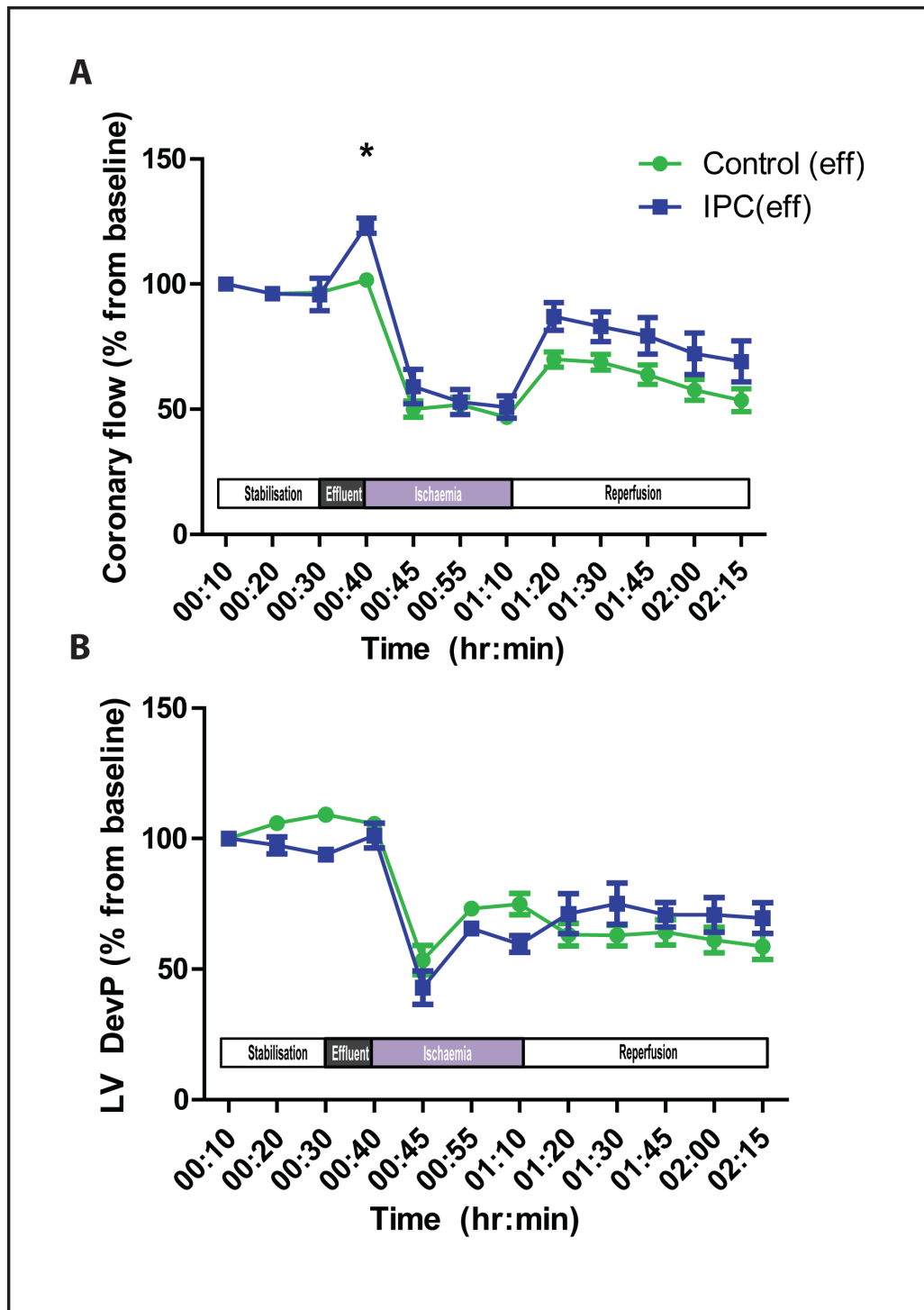
**Figure 3-6. Protein concentration in both control and IPC effluent.**

*Protein concentration in the effluent was analysed using the BCA method (section 2.6). No significant differences were found between the groups (IPC =  $72 \pm 7 \mu\text{g/ml}$  vs Control =  $81 \pm 9 \mu\text{g/ml}$ ,  $p = 0.43$ ).*



**Figure 3-7. Infarct size analysis of donor and recipient hearts.**

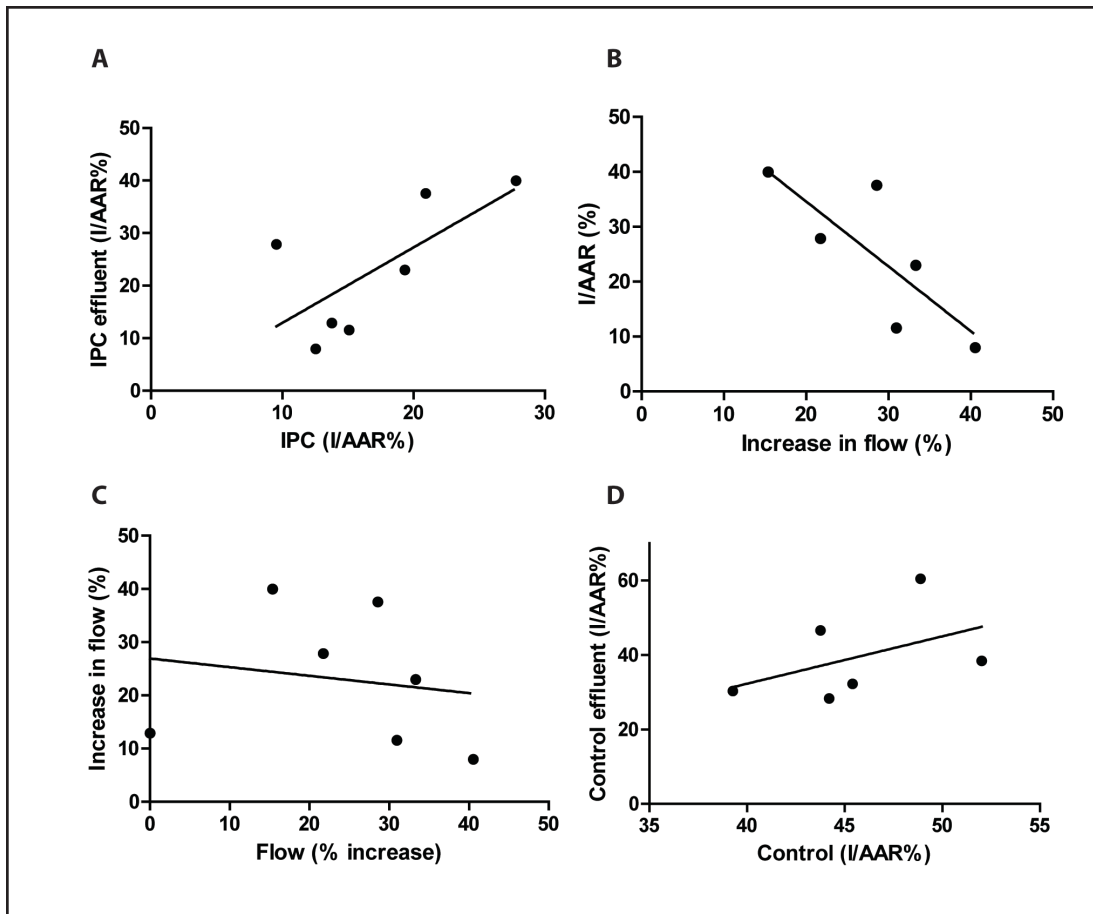
*Infarct size as measured by TTC staining revealed IPC effluent can induce significant cardioprotection to naïve isolated hearts (IPC effluent I/AAR % =  $17 \pm 3$  vs control effluent =  $42 \pm 2$ ,  $p < 0.05$ ). Hearts that effluent was collected from were subsequently infarcted; those hearts that received IPC were significantly protected relative to control (Donor IPC I/AAR % =  $22 \pm 6$  vs donor control =  $41 \pm 5$ ,  $p < 0.05$ ). Data presented as mean  $\pm$  SEM,  $n=6-8$  per group.*



**Figure 3-8. Functional parameters measured in hearts who received either control or IPC effluent.**

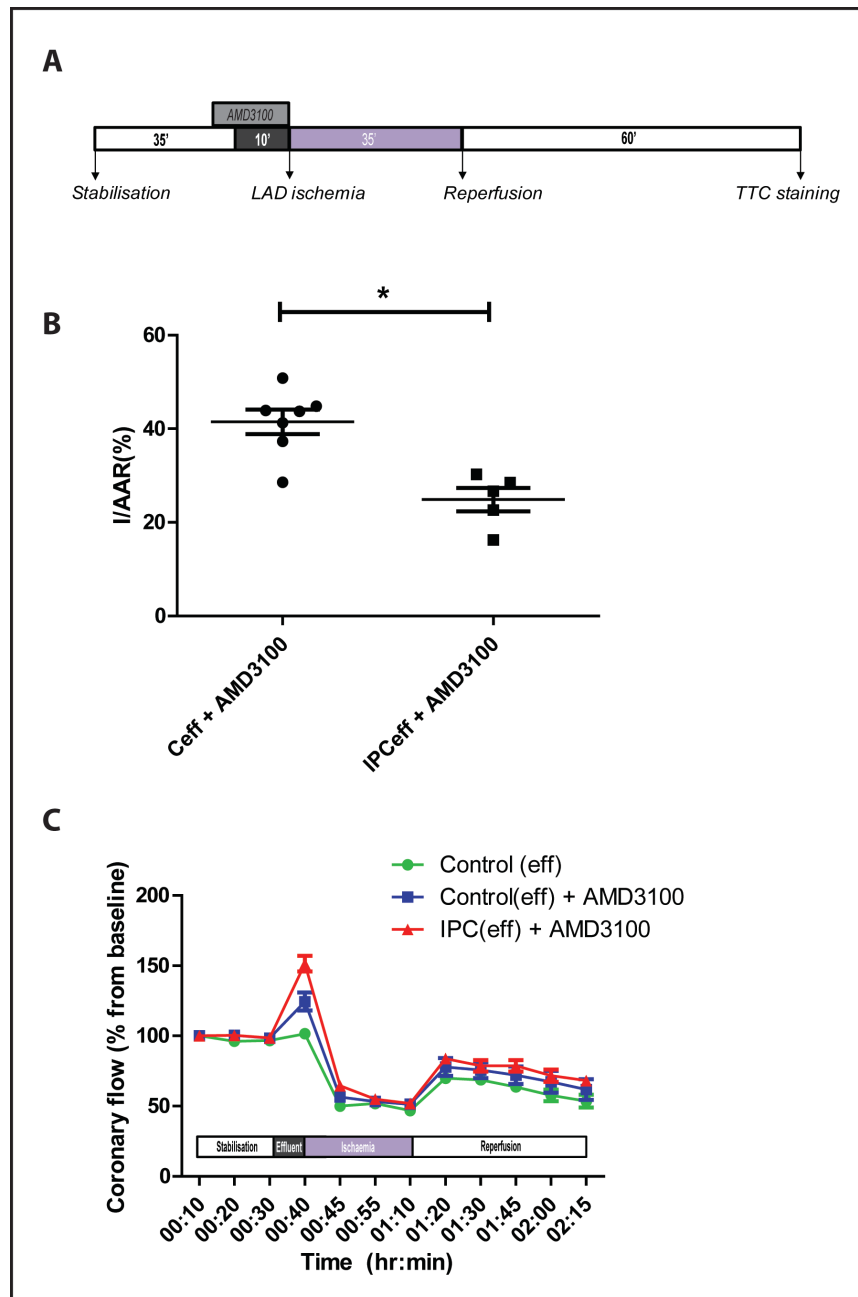
Measurement of (A) coronary flow rate and (B) left ventricular developed pressure, in naïve hearts that received control and IPC effluent, throughout the ischaemia-reperfusion protocol. All data presented as mean  $\pm$  SEM, data analysed by two-way ANOVA.





**Figure 3-9. Correlation between infarct size and flow rate indicate link between humoral factor and protection in acceptor and donor hearts.**

(A) Correlation between I/AAR% of IPC donor and acceptor hearts ( $r^2 = 0.48$ ,  $p=0.09$ ) (B) Correlation between increase in flow (%) in response to IPC effluent and the I/AAR% of IPC acceptor hearts ( $r^2 = 0.63$ ,  $p=0.06$ ), (C) correlation between increase in flow (%) in response to control effluent and the I/AAR% of control acceptor hearts ( $r^2 = 0.22$ ,  $p=0.29$ ), (D) correlation between I/AAR% of control donor and acceptor hearts ( $r^2 = 0.21$ ,  $p=0.36$ )



**Figure 3-10. Stromal-derived factor 1 alpha does not mediate the protection afforded by IPC effluent.**

(A) The antagonist for CXCR4, AMD3100, was perfused through the naïve heart for 5min prior to and the duration of effluent perfusion. (B) AMD3100 did not abrogate IPC effluent mediated cardioprotection. (C) Interestingly, AMD3100 alone seemed to induce vasodilation in the naïve heart, observed as an increase in coronary flow rate. Data presented as mean  $\pm$  SEM, data analysed by student's *t*-test (B) and repeated measures ANOVA (C).

### *SDF-1 does not mediate cardioprotection in this setting*

Figure 3-9A details the experimental protocol; briefly, the CXCR4 antagonist, AMD3100, was given to the naïve heart for 5min prior to and the duration of effluent perfusion. The drug did not abrogate the cardioprotection afforded by IPC effluent (IPC+AMD3100 I/AAR% =  $25 \pm 3$  vs Control +AMD3100 =  $41 \pm 2$ ,  $p > 0.05$ ) (Fig. 3-9B). Interestingly, AMD3100 alone appeared to have a vasodilatory effect on the heart, as observed by an increase in coronary flow rate (Fig. 3-9C). In addition, it augmented the increase in flow observed with IPC effluent.

### **3.4.4 Discussion**

This model demonstrates, in line with current literature, that the heart releases factors into the coronary circulation in response to ischaemic conditioning and that these factors, when perfused through a naïve heart prior to ischaemia-reperfusion, can limit cell death. This is observed as a reduction in infarct size in hearts that received IPC effluent, to a similar level as observed with direct IPC. Given IPC is thought to be a receptor-mediated effect, via release of small molecules such as adenosine and opioids, it is likely that the protection afforded by IPC in the donor heart will mirror the protection seen in the recipient heart. Indeed, the infarct sizes of the two groups are correlated, although non-significantly, which suggests the protective factors released during IPC contributes to protection in the donor heart as well as the recipient. The inherent variability in the Langendorff IRI model, in terms of infarct size, means that greater numbers are required to see a statistically significant correlation.

Functional recovery of the hearts at the end of reperfusion, as measured by LVDP and CFR, was improved in the IPC acceptor hearts, albeit non-significantly. Studies have reported varied effects of effluent on functional recovery [74, 75, 215]; indeed, it may not be a valid marker of cardioprotection at one hour of reperfusion, as it is impossible at this stage to differentiate between non-viable and stunned myocardium. In addition, the Langendorff model is not the ideal model to observe heart function, as the

heart is retrogradely perfused and no fluid moves through the left ventricle. Nonetheless, it further indicates a beneficial effect of IPC effluent.

Interestingly, the IPC effluent evoked a ~25% increase in CFR when perfused through the naïve heart (Figure 3-3-D), indicating the presence of a vasodilatory factor. The strength of the vasodilatory stimulus, as measured by increase in CFR, was correlated with the degree of protection afforded to the naïve heart. This may indicate that the vasoactive factor is responsible in part for effluent-mediated protection. The correlation was not observed with control effluent.

#### *Is inter-cardiac conditioning a valid model of RIC?*

When the model was first proposed, the authors drew similarities to the inter-organ conditioning observed *in vivo*. For example, it has been shown that brief ischaemia and reperfusion to the kidneys, mesentery and skeletal muscle *in vivo* could protect the myocardium from IRI. Thus, could this inter-organ protection be taken a step further, namely via transfer of effluent from one discretely perfused heart to another, or, indeed, from one animal to another? This hypothesis turned out true and the effluent model represents a remote form of cardioprotection, responsible for several important mechanistic insights into the phenomenon. The model is, however, based on too simplistic a view of RIC, that is, the idea that the protective factor is released from the conditioned limb. The RIC paradigm is more complex, involving both a neural and humoral pathway and, thus, cannot be accurately investigated using an isolated heart. An *in vivo* setting must be used to harness full potential of RIC reflex, which is why this project develops the dialysate model (section 3.5). Nonetheless, it is an interesting model of cardioprotection and, crucially, can be useful to investigate the factors released from the heart following IPC and the induction of protection in the acceptor heart. Indeed, this model is used in this thesis to investigate the involvement of intrinsic cardiac nerves and nitric oxide in the induction of protection.

### *Stromal-derived factor 1 alpha*

SDF-1 $\alpha$  is known to be cardioprotective, through its action on CXCR4 receptors and has recently been implicated in the mechanism of remote ischaemic conditioning. The CXCR4 antagonist, AMD3100, abrogated the efficacy of RIC in an *in vivo* model of acute myocardial infarction in rat [390]. This suggests SDF-1 $\alpha$  could be released following RIC and act as a humoral cardioprotective mediator [72]. However, AMD3100 did not abrogate IPC effluent-mediated protection, suggesting SDF-1 $\alpha$  is not released following IPC in the *ex vivo* rat heart.

The significance of this experiment is that it underlines the fact that this model is not an accurate representation of RIC. Rather, the effluent model highlights the humoral component to classical IPC, hence it can be used to study the identity of these factors and the mechanism of their release. Although SDF-1 $\alpha$  was not investigated further as part of this thesis, it was an important experiment as it revealed something of the nature of the effluent model.

### 3.5 Aim 3: Establish a model of remote ischaemic conditioning via transfer of plasma dialysate

Some of the data presented in this section was recently published

**Pickard JMJ, Davidson SM, Hausenloy DJ, Yellon DM (2016)** *Co-dependence of the neural and humoral pathways in the mechanism of remote ischaemic conditioning. Basic Res Cardiol 111:50. doi: 10.1007/s00395-016-0568-z*

#### 3.5.1 Background

The unique mechanistic trait of RIC is the communication of a protective message from the conditioned limb to the myocardium and beyond. Initial studies suggested this occurred via release of a cardioprotective factor into the blood, however it became clear that a neural component was also important. How these two fit together within the RIC reflex was not immediately apparent; that is, are they mechanistically distinct, or do they interact with one another? With this in mind, the Redington group developed a model of RIC using a combination of *in vivo* and *ex vivo* techniques. Briefly, RIC was performed *in vivo* on rabbits, at which point a large volume of venous blood was sampled and centrifuged to isolate the plasma. The plasma was then dialysed across a <15 kDa membrane into a 20-fold dilution of KHB. This dialysate was then perfused through a naïve isolated rabbit heart prior to IRI, the hypothesis being that if a protective factor is released into the blood following RIC *in vivo*, it will be detected as a reduced infarct size *ex vivo*.

Although this model is based on the assumption that the humoral mediator is the endpoint in the communication phase of RIC, it enables investigation into the regulation of its release. The first startling discovery this model gave was the dependence of the humoral pathway on an intact neural pathway to the limb. Indeed, femoral nerve section or stimulation abrogated or induced release of the humoral factor following RIC respectively. Similar results could be obtained via intra-arterial adenosine, which again were inhibited via

femoral nerve section. Moreover, pretreatment with the nitric oxide donor S-nitroso-N-acetylpenicillamine (SNAP) abrogated release of the factor, induced by both RIC and i.a. adenosine. Taken together, these data suggest RIC induces local release of adenosine that, via activation of NO-sensitive sensory afferent nerves, induces release of the humoral factor.

The type of sensory nerves activated following RIC have been further characterised, based on the observation that both topical capsaicin and transcutaneous electrical nerve stimulation (TENS) induce release of a protective factor into the blood, with a similar effect to that observed following RIC. Thus, it was suggested that activation of small diameter (A $\delta$ - or C-fibre) afferents was essential for initiation of the RIC reflex and for subsequent release of the humoral factor into the blood [248, 290].

These three studies were the first to demonstrate an interaction between the humoral and neural pathway. Since corroborated in humans [164], it seems a reproducible scientific observation that pervades several species. The key question that precipitates, however, is how sensory afferent nerve activation induces release of the humoral factor. The aim of this section was to establish and validate the dialysate model for the first time using Sprague-Dawley rats. This thesis aims to use the model to elucidate the link discussed above, with particular emphasis on the vagus nerve, whose activation has been implicated in RIC [51, 79, 174, 243, 278].

### 3.5.2 Methods

Detailed methods can be found in section 2.3.

Male Sprague-dawley rats were anaesthetised and prepared as described in section 2.3.1. Subsequently, they had a small cuff placed around their right hindlimb and were randomised to receive one of two interventions: (1) Remote ischaemic conditioning (RIC) protocol, consisting of 4 x 5 min cuff inflation to 200 mmHg with intermittent 5 minute deflations, or, (2) sham protocol, consisting of a corresponding time period with no cuff inflation (see Figure 3-11). Immediately following each intervention, the animal was sacrificed and blood sampled via right ventricular puncture. The plasma was subsequently centrifuged to obtain platelet-free plasma, and dialysed across a 12-14kDa membrane for 24hr at 4°C. The dialysate was then perfused through a naïve isolated rat heart for 10min prior to a 35min left anterior descending coronary artery ischaemia and 60min reperfusion.

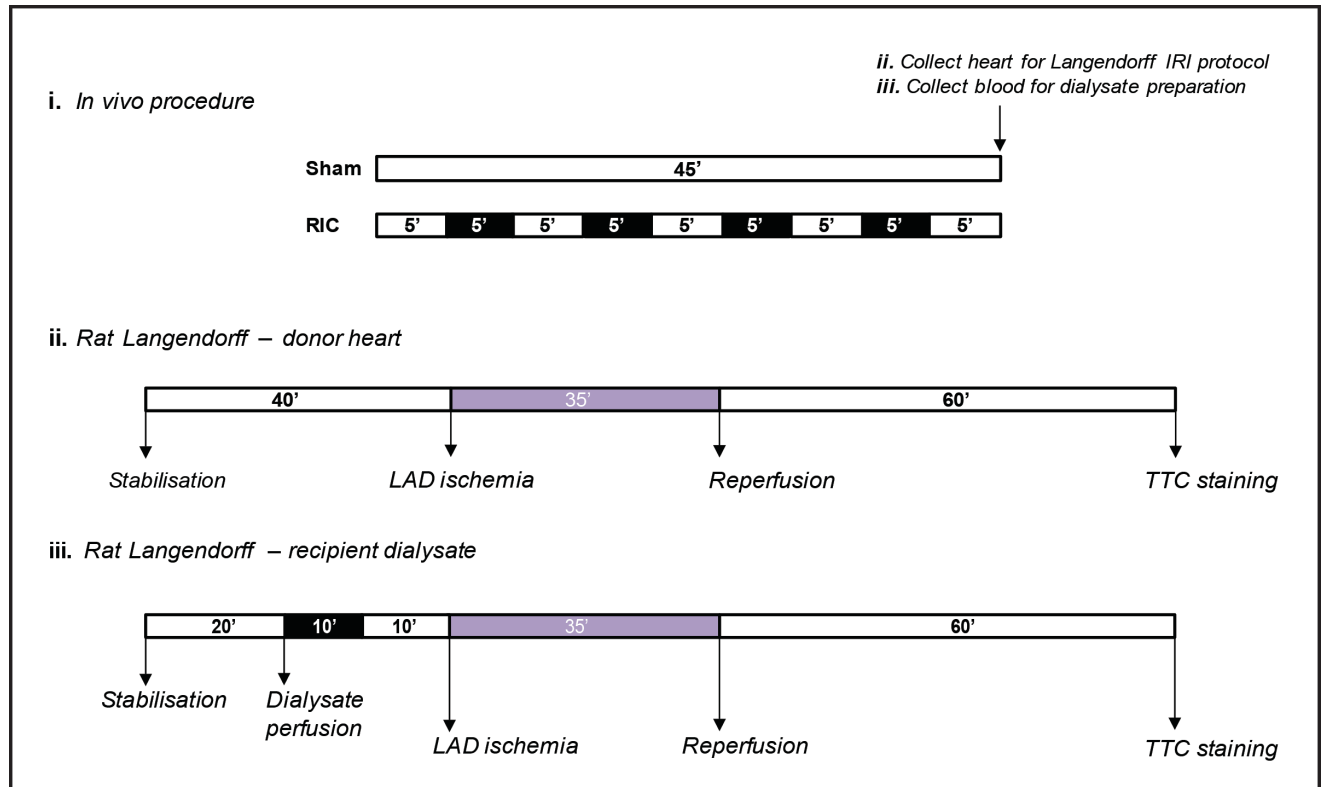
In the initial experiments, platelet rich plasma was used for dialysis. However, it became clear that platelets were confounding the observed effect, thus platelets were subsequently removed from the plasma via an extra centrifugation step prior to dialysis.

#### *Experimental endpoints*

1. The primary endpoint was infarct size, expressed as a percentage of the area-at-risk. This was performed at the end of reperfusion using Evans blue and triphenyl tetrazolium chloride staining, as described in section 2.2.5.
2. Several functional endpoints were also measured in the naïve isolated heart throughout the experiment. Pressure and heart rate measurements were taken via a fluid-filled balloon in the left ventricle. In addition, coronary flow rate was manually measured at set time points throughout the protocol. These are described in more detail in section 2.2.3.



3. Dialysate was analysed for protein concentration using the BCA method, described in section 2.6.



**Figure 3-11: Protocol for *in vivo* RIC or sham procedures:**

Anaesthetised rats were subjected to either (1) RIC procedure, consisting of 4 x 5 min hindlimb ischaemia (■) with intermittent 5-minute reperfusion (□) or, (2) sham procedure, consisting of a time-matched period of anaesthesia. (ii) Following *in vivo* RIC or sham procedures, hearts were excised and perfused on a Langendorff system. Isolated perfused rat hearts were, after a 20-minute stabilisation period, perfused with either control or RIC dialysate for 10 minutes. After a 10 minute washout period, the hearts were put into ischaemia via LAD occlusion for 35 minutes, followed by 60 minutes of reperfusion.

### 3.5.3 Results

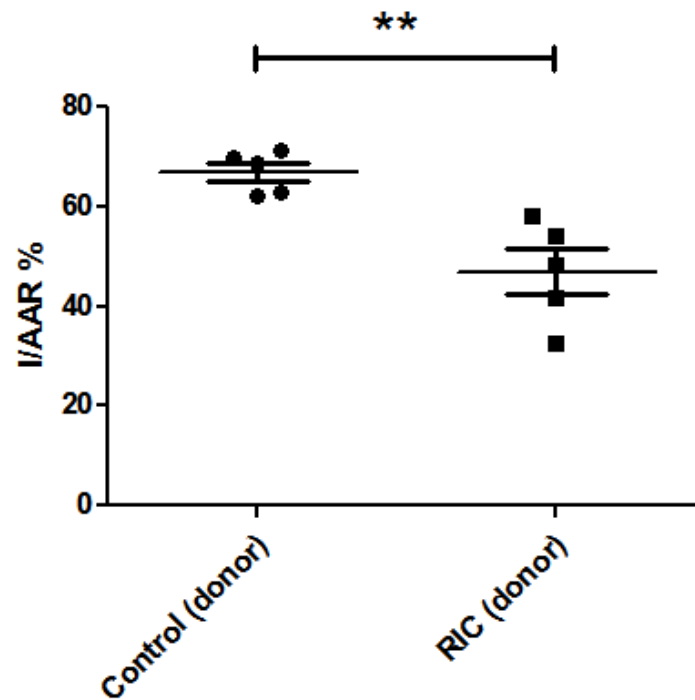
It was important, initially, to confirm that the RIC stimulus given *in vivo* could protect the recipient heart from IRI. Therefore, after the blood was sampled for dialysate preparation, the heart was excised, perfused on a Langendorff rig and subjected to a 35min LAD occlusion and 60min reperfusion. Those hearts that received *in vivo* RIC demonstrated a significantly reduced infarct size (I/AAR=46.9 ± 4.6%, n=5, p<0.01 vs control) relative to control (I/AAR=66.9 ± 1.9%, n=5).

The results of the first set of dialysate experiments are shown in Figure 3-13. Hearts that received both control and RIC dialysate displayed a cardioprotective phenotype, with infarct sizes of 26.3 ± 1.0% (n=3) and 16.7 ± 1.5% (n=4) respectively. Although RIC dialysate offered statistically significant additive protection relative to control (p<0.01), it seemed there was a degree of artefactual cardioprotection conferred as a result of the experimental design, since the infarct sizes in the control group were smaller than expected.

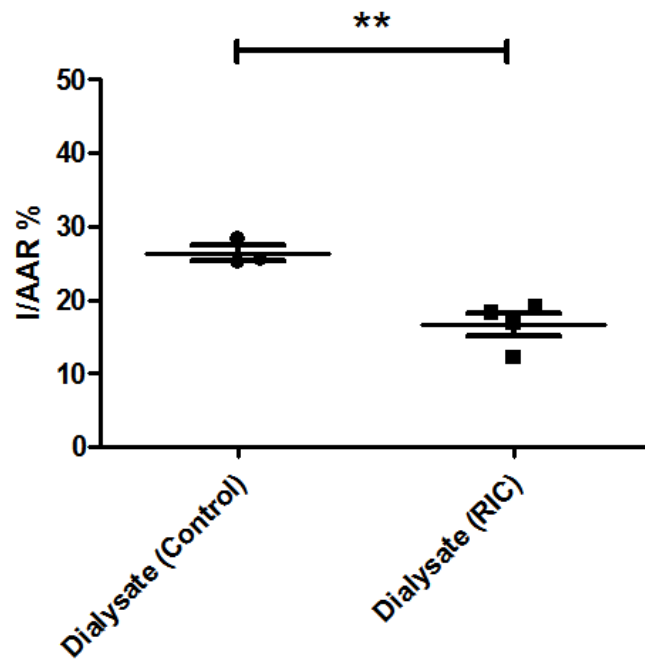
It is known that platelet activation, or indeed inhibition, can induce cardioprotection. The alpha-granule is known to contain several cardioprotective proteins, including sphingosine-1-phosphate and stromal-derived factor 1 alpha (SDF-1α). Crucially, activation and degranulation of platelets due to hypothermia has been well described.

Thus, it was hypothesised that during dialysis the platelets would degranulate and release the cardioprotective factors, which in turn move into the dialysate and could induce the artificial cardioprotection observed in Figure 3-13. To test this, the platelets were removed from the plasma via an additional centrifugation step at 10,000 g for 30 minutes at 21°C. The dialysis was performed exactly as before, and then perfused through a naïve heart prior to IRI. The artificial protection observed in Figure 3-13 was no longer present; hearts that received RIC dialysate had a significantly reduced infarct size (I/AAR=27.6 ± 2.3%, n=7, vs control I/AAR=42.9 ± 1.2%, n=8, p<0.001). The control infarct size was very much in line with the literature for the IR

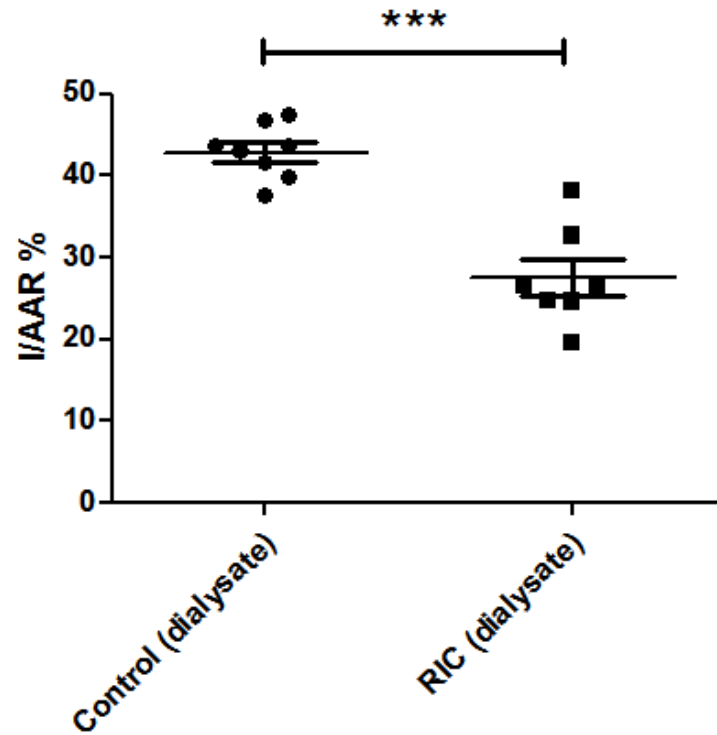
protocol, and much larger than was observed when platelet-rich plasma was used.



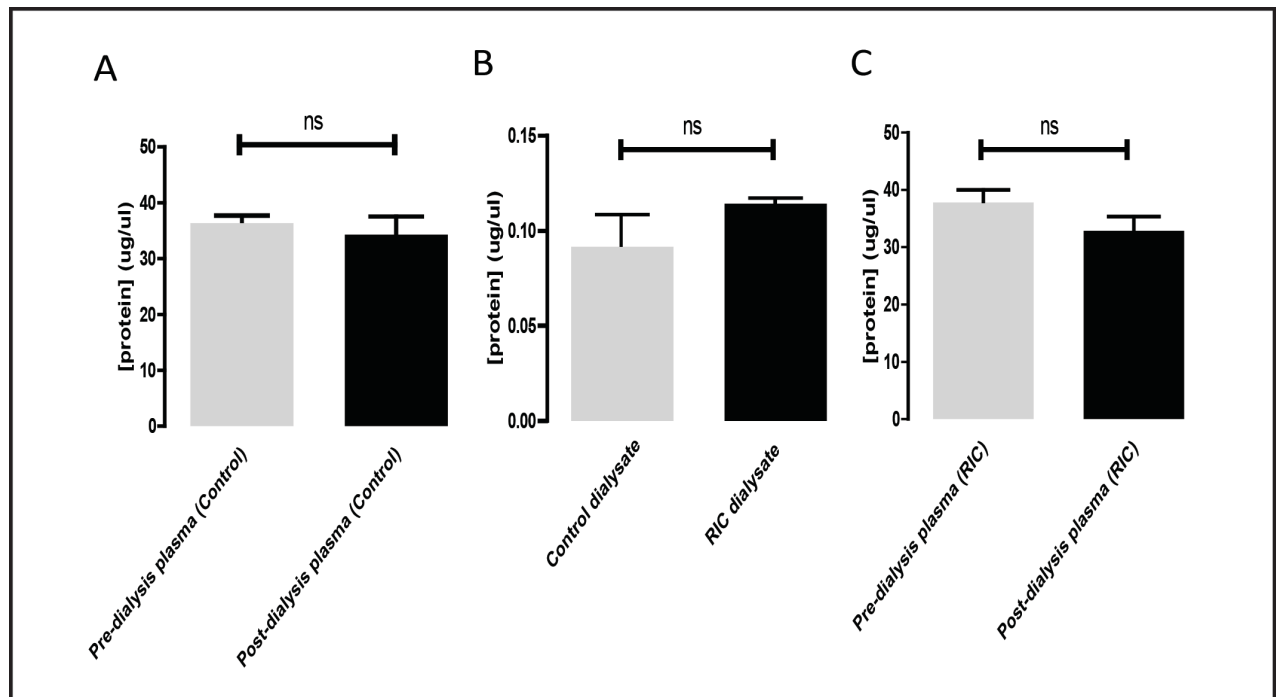
**Figure 3-12: In vivo RIC significantly reduces infarct size of the donor heart:** Rats were anaesthetised and randomised to receive either in vivo RIC or sham, hearts were excised and perfused on a Langendorff rig. The hearts were then subjected to 35-minute LAD occlusion and 60-minute reperfusion. Statistical analysis was performed via an unpaired, two-way student's t-test,  $n=5$  per group.



**Figure 3-13: Platelet-rich plasma creates a dialysate that, for both control and RIC, induces a cardioprotected phenotype.** Although RIC dialysate offered a significantly reduced infarct size relative to control, both dialysates seemed to induce a cardioprotected phenotype in the naïve hearts. This is likely artificial protection, indicating the presence of a systematic error.

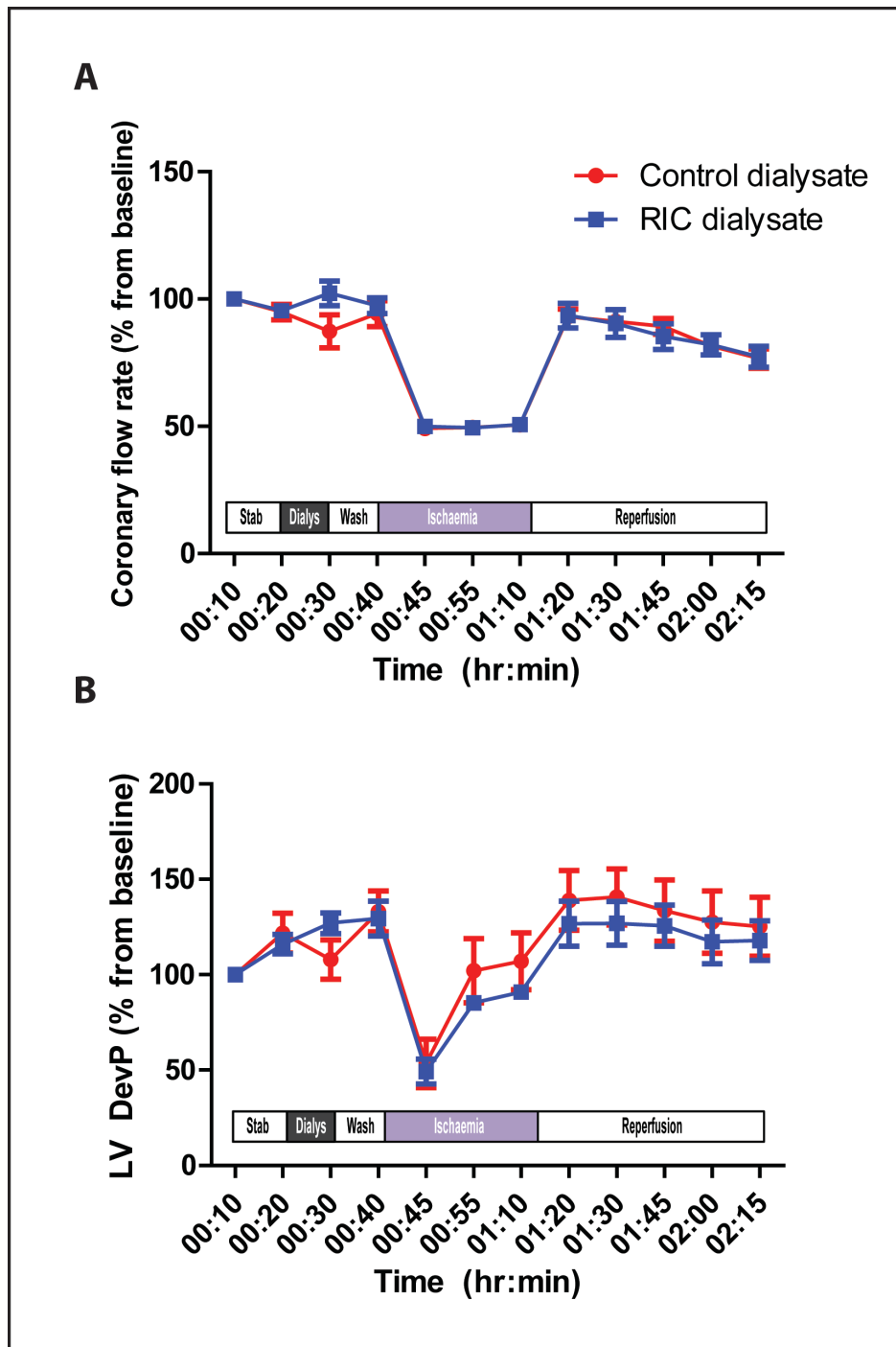


**Figure 3-14: Removing platelets from the plasma abolishes the artificial protection and reveals the significant cardioprotection associated with RIC dialysate.** Hearts that received RIC dialysate were significantly protected from IRI relative to control (I/AAR=27.6  $\pm$  2.3%, n=7, vs control I/AAR=42.9  $\pm$  1.2%, n=8, p<0.001). Removing platelets from the plasma seemed to abolish the artificial protection seen previously, indicating that platelet activation was occurring and releasing a factor into the dialysate which contributed to the protection.



**Figure 3-15. Protein concentration in dialysate and plasma following RIC or sham.**

(A-C) BCA protein assay was employed to measure protein concentration in the plasma following either RIC (4x5min hindlimb ischaemia/reperfusion) or sham surgery. No difference in plasma concentration was observed between pre- and post-dialysis groups, and no difference was observed following RIC relative to sham. Interestingly, a 400-fold reduction in protein concentration was observed within the dialysate when compared to plasma.



**Figure 3-16. Functional parameters measured in the naïve hearts who received dialysate.**

(A) Coronary flow rate of the naïve recipient hearts. No significant increase in functional recovery from ischaemia was observed in hearts that received RIC dialysate. (B) Left ventricular pressure in the naïve heart as measure via insertion of a fluid filled balloon. Again, no significant improvement in recovery from ischaemia was observed in hearts that received RIC dialysate. Data expressed as mean $\pm$ SEM, and analysed using two-way ANOVA.

### 3.5.4 Discussion

We have demonstrated that a plasma dialysate generated following *in vivo* RIC in rats is able to significantly protect a naïve-isolated rat heart from ischaemia-reperfusion injury. Previous studies have prepared dialysate from an *in vivo* rabbit model [248, 289, 331] or indeed humans [144, 164, 320]. These used a 20-fold dilution gradient into the dialysate, which is 2.5-fold lower than the 50-fold dilution used in this study. This indicates both the conserved nature of the RIC mechanism across several mammalian species and the remarkable potency of cardioprotection offered by the factor(s).

The unusual observation here is that platelets seem to release one or more factors into the dialysate, which induce cardioprotection. When platelets were removed from plasma, the artificial protection observed in Figure 3-13 was abolished. This is highlighted when one considers the control infarct sizes for platelet-rich (I/AAR=  $26.3 \pm 1.0\%$ ) and –poor (I/AAR=  $42.9 \pm 1.2\%$ ) plasma dialysates when perfused through a naïve heart. It is known that platelets become activated at low temperatures [91, 175, 402], thus it was hypothesised that during the dialysis, which took place at 4°C, platelets would activate and degranulate, releasing many proteins into the plasma, some of which would diffuse across the membrane into the dialysate. Alpha-granules of platelets contain many known cardioprotective proteins, including insulin-derived growth factor 1 (IGF-1) [171], sphingosine-1-phosphate [194], stromal-derived factor-1-alpha (SDF-1 $\alpha$ ) [241] and adenosine [383]. Indeed, unpublished data from this laboratory has demonstrated that platelet activation induces a large release of SDF-1 $\alpha$  into plasma.

The evidence surrounding platelets and cardioprotection, however, is contentious. Perfusion of isolated rat hearts with rat platelets reduced injury from IR, via release of factors including serotonin and adenosine [382–384]. However, several studies have demonstrated P<sub>2</sub>Y<sub>12</sub> antagonism, therefore presumably inhibiting platelet activation, induces cardioprotection in an *in vivo* model of IRI [63, 385, 386]. Thus, it is not clear where the observation in Fig. 3-12 fits in with the literature. The simplest explanation, however, is that



activation of platelets in the plasma releases factors into the dialysate that induce cardioprotection. Whether platelet activation is a component of RIC communication is unknown; this thesis does not focus on the answer to this question.

The great advantage of this model is it allows definition between the different components of the RIC reflex:

- *Trigger.* At the *in vivo* level, one can intervene prior to RIC either pharmacologically or surgically. This has produced interesting insights into the trigger of RIC. For example, the observation that release of the humoral factor occurs downstream of sensory nerve activation in the conditioned limb was a paradigm shift in the field, demonstrating for the first time the interdependence of neural and humoral pathways [331].
- *Communication.* The model facilitates characterisation of the humoral factor released into the blood following RIC. Several studies have attempted to do so via either proteomics or fractionation of the plasma. Whilst proteomic studies have proved fruitless thus far [138, 139, 143], fractionation of the plasma has suggested the factor is hydrophobic, resistant to freezing and thawing, not easily denatured and less than 15kDa [320].
- *Induction of cardioprotection.* One can add various antagonists to the dialysate to investigate the mechanisms of initiation of cardioprotection. This can be at the level of receptor activation on the myocyte, or activation of intracellular signalling pathways. These mechanisms have not been well described for RIC; this project aims to elucidate some of them.

Despite it being a useful and valid model of RIC, there are several limiting aspects of the study design, which must be considered. First, the choice of a 12-14kDa membrane for the dialysis was, although based on previous literature, arbitrary. Studies using the effluent model have used dialysis to suggest a cardioprotective factor exists between 3.5 and 30kDa [43, 317].

Thus, the Redington group used the 12-14kDa cut-off when they were developing the dialysate model, with the assumption that inter-cardiac conditioning was mediated via similar factor(s) as *in vivo* limb preconditioning. Although this is not an unreasonable assumption, it is made without evidence as the two models cannot be absolutely compared. Thus, it is not clear whether there is a factor greater than 15kDa, which also contributes to protection. Indeed, a recent study implicated apolipoprotein A1 (Apo-A1) in RIC; this protein is significantly larger than 15kDa [142]. Second, the blood sampling technique is different to that of previous dialysate models using rabbits. Whereas in rabbits blood was sampled via a vein in the ear, in rats the blood was aspirated via right ventricular puncture. This is a very invasive procedure, thus perhaps the trauma itself was causing release of protective factors into the plasma, contributing to the dialysate-protection. It is known that surgical trauma can induce cardioprotection, via activation of nociceptive c-fibre afferents [112]. Third, the model is based on the assumption that the humoral factor is the end effector of communication. It does not rule out the possibility that there is an additional neuronal phase upstream of the humoral phase, or indeed the possibility that the humoral messenger is the product of neurotransmitter spill-over. This is discussed in more detail in chapter 5.

The potential of this model has not yet been fully realised with respect to RIC, with only a handful of studies employing its use thus far. There are several fundamental questions surrounding the RIC paradigm which the model can help to answer; namely, the link between sensory nerve activation and production of the factor and, crucially, the source of the humoral factor. This thesis aims to utilise the model and shed light on the above questions.

### **3.6 Summary**

This chapter detailed the characterisation of the Langendorff isolated perfused heart model and, subsequently, two models of cardioprotection. The work detailed above comprises the majority of the year of work undertaken for this thesis. Once characterised, they provided a platform from which to investigate the mechanisms of cardioprotection, both in vivo and ex vivo.

The first, a model of inter-cardiac conditioning via transfer of preconditioned coronary effluent, first demonstrated by Dickson et al. [75]. Whilst originally presented as proof of a humoral mediator of remote ischaemic conditioning, it is more representative of the humoral component to classical ischaemic conditioning. This model is used in chapter 7 to investigate the importance of the intrinsic cardiac nervous system in classic IPC.

The second model was adapted from that first presented by Shimizu et al [320], designed to investigate the release of a humoral blood-borne cardioprotective factor. This model allowed investigation into the mechanism of release of the humoral factor, and its mechanism of action on the heart. The model is used to this effect in chapters 5 and 6.

## Chapter 4 REMOTE ISCHAEMIC CONDITIONING REDUCES INFARCT SIZE IN ANIMAL IN VIVO MODELS OF ISCHAEMIA-REPERFUSION INJURY: A SYSTEMATIC REVIEW AND META-ANALYSIS

This chapter details a collaborative project, with a manuscript co-authored with Dr Daniel Bromage [45]. The study was conceived and designed with Dr D. Bromage. Mr N. Burke and Dr O. Ziff collected the data for analysis of study quality. All other data was collected and the manuscript co-written with Dr D. Bromage. The figures were co-prepared with Dr X. Rossello. The statistical method was co-designed with Dr X. Rossello and executed in its entirety by him. All contributors were from the Hatter Cardiovascular Institute (UCL, UK). A version of this study can be found in the thesis of Dr Daniel Bromage, although significant changes have been made in line with subsequent referee comments. Chapter 10 contains a copy of the published manuscript.

***Bromage DI \*, Pickard JM \*, Rossello X, Ziff OJ, Burke N, Yellon DM, Davidson SM (2016) Remote ischaemic conditioning reduces infarct size in animal in vivo models of ischaemia-reperfusion injury: a systematic review and meta-analysis. Cardiovascular Research (In Press)***

\* = Authors contributed equally

### 4.1 Introduction

Myocardial ischaemia-reperfusion injury describes the deleterious consequences of several pathological processes and cardiac interventions. Most commonly, it is caused by thrombotic occlusion of the coronary artery in ST-segment elevation myocardial infarction (STEMI) and subsequent reperfusion by primary percutaneous coronary intervention (PPCI), but it may result from a range of elective and emergent causes of myocardial ischaemia, including cardiopulmonary bypass and spontaneous reperfusion of STEMI. Despite constantly improving medical and surgical practice, myocardial IRI remains associated with significant morbidity and mortality.

For example, in STEMI and despite PPCI, 30-day, 1-year, and 5-year cardiac mortality remains 7.3 %, 8.4 %, and 13.8 %, respectively [269].

As discussed in Chapter 1, the myocardium can be protected from lethal IRI by the application of multiple brief cycles of ischaemia and reperfusion to an organ or tissue remote from the heart, before, during, or after the index ischaemia (preconditioning, perconditioning or postconditioning, respectively). Limb remote ischaemic conditioning (RIC) is a cheap, non-invasive intervention that, since its inception in 1997 [35], has been successfully demonstrated in several pre-clinical studies of myocardial IRI. Subsequently, several phase II, proof-of-concept clinical studies have translated these findings to variety of clinical settings, albeit frequently using cardiac enzymes as surrogate markers of cellular injury, including coronary artery bypass surgery (CABG) [114, 128, 339, 350], elective abdominal aortic aneurysm repair [5, 358], elective cervical decompression surgery [154], elective PCI [151], and in PPCI for STEMI [41, 184, 311].

However, more recent, large clinical-endpoint studies of RIC in cardiac surgery have been neutral [122, 249]. Although cardiac surgery may be an inappropriate setting for RIC, given the small peri-operative injury and lack of injurious warm ischaemia-reperfusion [99], these findings have prompted an interrogation of the pre-clinical evidence base for RIC and a perceived lack of systematic pre-clinical characterisation of the optimal RIC stimulus. This is in contrast to direct ischaemic conditioning that, despite being limited by the necessity to intervene before the index ischaemia, has been thoroughly characterised [368].

## **4.2 Research aims and objectives**

Well-designed animal studies can provide useful information on relevant factors that may influence outcome. We performed a comprehensive systematic review and meta-analysis to scrutinize basic studies of RIC in *in vivo* animal models of myocardial IRI. Our aim was to ascertain the overall effect and variability of RIC in this context, compared to control (sham procedure or no treatment). We further aimed to investigate determinants of efficacy, including variables such as RIC protocol and use of supplementary oxygen. Our hypothesis was that study quality and publication bias would result in over-estimation of the effect size associated with RIC [153, 211].

## **4.3 Methods**

### **4.3.1 Systematic review**

The systematic review was performed in accordance with Preferred Reporting Items for Systematic reviews and Meta-Analyses (PRISMA) guidelines [221].

#### **4.3.1.1 Literature search strategy**

A literature search was conducted on 21<sup>st</sup> August, 2015 by JP. The search strategy was defined in an iterative manner, using previously published guidelines [95, 237, 238], by DB and JP and peer reviewed by members of the Hatter Cardiovascular Institute research group. Our search will be limited to reports available in English due to limited time and financial resources for translation. We will use the following search strategy, incorporating keywords and MeSH terms, for MEDLINE:

1. (remote adj2 \$condition\$).mp.
2. (remote adj isch\$emic).mp.
3. remote precondition\$.mp.
4. remote postcondition\$.mp.
5. remote percondition\$.mp.
6. limb \$condition\$.mp.
7. ripc.mp.
8. limb isch\$emi\$.mp.
9. \$condition\$.mp.
10. 8 and 9
11. 1 or 2 or 3 or 4 or 5 or 6 or 7 or 10
12. exp Reperfusion Injury/
13. exp Myocardial Ischaemia/
14. Myocardial Infarction/
15. infarct size.mp.
16. ("infarct size" or "size of infarct\*").mp.
17. cardioprotection.mp.
18. 12 or 13 or 14 or 15 or 16 or 17
19. 11 and 18
20. Limit 19 to animal
21. Limit 20 to (english language and yr="1997-Current")
22. Limit 21 to review articles
23. 21 not 22

Differences in the search strategy between Medline and Embase are described in table S1.

Search term	Database	
	Medline	Embase
Cardioprotection	cardioprotection.mp. (keyword)	heart protection/ (MeSH term)
Search review articles	limit 21 to review articles	limit 21 to "reviews (maximizes sensitivity)"

**Table 4-1. Differences in search strategy between databases**

Lastly, transplanted hearts were added to the exclusion criteria *post hoc* as it was apparent that these experiments fulfilled our inclusion criteria but were likely to confer additional injury during the transplantation process.

#### **4.3.1.2 Study selection**

Study eligibility criteria were defined using the PICOS approach [221]. *In vivo* animal studies were included and were eligible if they investigated the effect of limb RIC (pre-, per- or post-) versus a control (sham procedure or no treatment) on myocardial infarct size, as measured using tetrazolium chloride (TTC) [30], in any mammalian species, regardless of study design. Transient infra-renal aortic occlusion was considered as bilateral hind limb ischaemia.

Studies were excluded if they did not include or report absolute myocardial infarct size (IS) as a percentage of area at risk (AAR, defined as the myocardial tissue within the vascular territory that is distal to occluded artery and, if not reperfused, is at risk of irreversible ischaemic death) [296]. The AAR varies depending on the exact position of the LAD suture and variable LAD anatomy. Infarct size has a strong positive correlation with AAR and therefore, without correction, a small AAR could create false-positive results for cardioprotection, and vice versa [392]. Furthermore, studies were excluded if they specifically investigated only the 'second window' of cardioprotection (RIC to infarction interval > 1 h) [206, 240], if RIPostC was initiated more than 10 min after reperfusion (after which it is generally believed unlikely to be effective) [24, 262], if the animals had co-morbidities, if infarct size was only measured using a method other than TTC, or if they investigated the impact of RIC in the context of heart transplant. Groups in which RIC was administered in combination with another conditioning protocol (local conditioning, for example), or with pharmacological treatments known to have cardioprotective effects, were excluded. Finally, studies investigating neonatal animals were excluded to ensure clinical relevance to IRI.

Reports were excluded if they were not available in English and a publication date restriction of 1997-present was imposed in view of the first publication of



the efficacy of RIC in the limb [35]. Review articles, abstract articles, unpublished material and ongoing studies were excluded.

Retrieved records were screened for eligibility using the title and abstract, followed by the full text. Eligibility assessment was performed independently in an un-blinded, standardized manner by 2 reviewers (DB and JP). To ensure reliability and improve subjectivity of screening of relevant titles and abstracts, DB and JP first independently screened 10% of a random sample of the search results. The exercise was repeated until 90% agreement was achieved, prior screening all search results. Disagreements between reviewers were resolved by examining the full text of the article or by consensus. When this failed to resolve the disagreement, attempts were made to contact authors of the original study by e-mail, where appropriate. Further unresolved conflict was resolved by arbitration from DB. The full text of eligible records was retrieved and subjected to full text screening. DB and JP performed the same calibration exercise as described prior to un-blinded, standardized eligibility assessment. Conflicts were resolved as per the title and abstract screening.

During the title and abstract screening calibration exercise, inter-rater agreement was 94%, with no instances of arbitration or reason to contact the authors of the study. During full text screening, inter-rater agreement was 87%, with no instances of arbitration or reason to contact the authors of the study. The 13% of references upon which the author disagreed were accounted for by screening errors rather than fundamental disagreement about the features of the study.

#### **4.3.1.3 Data extraction**

We have developed a data extraction sheet based on the Cochrane Consumers and Communications Review Group's data extraction template [221], which was be pilot-tested on ten randomly-selected included studies, and refined accordingly.

Data were independently extracted by 2 authors (DB and JP) using predefined data fields, including study quality indicators. To ensure reliability

and improve subjectivity of data extraction, DB and JP independently collected data from 10% of a random sample of included studies. The exercise was repeated until 90% agreement is achieved, before extracting data from all included studies. Disagreements were resolved by consensus. When this failed, attempts were made to contact authors of the original study by e-mail, where appropriate. Further unresolved conflict was resolved by arbitration from DB.

Following data extraction, searches were conducted based on first and senior author name, sample size and size of outcome to identify double counting. These parameters were chosen due to expected high completeness and based on recommendations in the PRISMA statement [221]. If multiple reports for the same study are identified they were compared for logical inconsistencies, and subsequently were accounted for by contacting the report author by e-mail. We likewise attempted to acquire key missing information in the same way.

#### **4.3.1.4 Data items**

Variables for which data were sought were developed using the PICOS approach. Data items were chosen according to experimental variables with evidence for an effect on myocardial IRI as these were considered likely to impact on the efficacy or RIC, and variables that we considered of potential importance. Examples of such variables are species and strain, gender, choice of anaesthetic and duration of ischaemia and reperfusion (**Table 3-2**).

We also included variables without extensive evidence that we consider of potential importance. Although not all the data items have been investigated in the literature in the setting of cardioprotection, we aimed to cover a broad range of experimental variables in order to obtain a clear picture of how the efficacy of RIC may be influenced *in vivo*. We have not performed a separate systematic review but we believe we have accounted for all potentially pertinent methodological variables. Any data items added after the review started will be highlighted and justified. All extraction forms will be archived and made available, if required.

We also made the following assumptions:

- The included studies contained several definitions of pre-, per- and post-conditioning. For clarity, despite the terminology used in the report, we defined preconditioning as any stimulus where the last cycle of ischaemia had been completed by the time of index ischaemia, perconditioning as any ischaemic stimulus that overlapped in full or in part with the index ischaemia, and postconditioning as any stimulus that began at or after the time of myocardial reperfusion.
- When a report described 'femoral artery occlusion' we assumed this to be with a vascular clamp. Likewise, where hindlimb occlusion was reported we assumed the use of an external cuff or tourniquet. A cuff was defined as an external inflatable device, while a tourniquet described any other kind of external compression. In all cases we assumed the cuff was inflated to an appropriate pressure.
- We assumed any reperfusion duration  $\leq 4$  h to be non-recovery and anything over that to be a recovery model.
- We defined a time of  $>1$  hr from the last RIC cycle to the onset of index ischaemia as the 'second window' of protection. This study was concerned with the acute cardioprotective potential of RIC, thus these studies were excluded from the analysis.
- Descriptions of the LAD and left coronary artery (LCA) were assumed to be interchangeable in rodents.

Category	Data item
Study details	First author, senior author, date, report format (abstract/full), country of origin
Populations/subjects (P)	Species, strain, age/weight
Interventions (I)	Pre-/per-/post-conditioning, RIC cycle time, RIC number of cycles, number of limbs, RIC technique (cuff/clamp), time to index ischaemia, index ischaemic duration, coronary territory occluded, reperfusion duration, non-recovery vs. recovery, anaesthetic type, anaesthetic dose (mg/kg), other peri-operative medication, use of anti-coagulant, type of anti-coagulant, intubated/ventilated, supplementary oxygen
Outcomes (O)	Infarct size as primary endpoint, mean infarct size (%AAR), mean AAR, AAR technique, standard deviation, SEM, other reported statistical analyses (pertinent to infarct)
Study design (S)	Sample size

**Table 4-2. Data items, developed using the PICOS approach**

#### **4.3.1.5 Risk of bias**

We used a component approach to assess study quality, based on the study report, using the ‘**Animal Research: Reporting of *In Vivo* Experiments**’ (ARRIVE) guidelines [187, 247]. We also used a separate 12-item score, adapted from that developed by Macleod *et al.* in response to a perceived failure of translation of promising neuroprotective agents in stroke [236]. We removed two and added 4 items, resulting in a 12-item custom score which we applied to studies in the meta-analysis. If the monitoring of ST segments or rhythms was reported, we assumed that heart rate monitoring as available.

Added to Cochrane study quality score	Removed from Cochrane study quality score
Statement of measurement of PaO <sub>2</sub> and SaO <sub>2</sub>	Use of animals with co-morbidities
Statement of recording of ECG	Blinded induction of ischaemia
Statement of measurement of blood pressure	
Blinded application of conditioning protocol	

**Table 4-3. Items removed and added from the Cochrane study quality score to make the adapted score used in the study.**

Study quality was assessed independently from data extraction and between assessors in an un-blinded, standardized manner by 2 reviewers (OZ and NB). Disagreements were resolved by consensus in all cases. We assessed the relationship between study quality and the overall effect of RIC on IS/AAR% using meta-regression, as described above.

Potential publication bias was assessed by visual inspection of a funnel plot for asymmetry, and Egger's regression analysis for small study effects. No protocols were available with which to examine for selective reporting; however, the methods and results sections of all included studies were carefully compared for inconsistencies.

#### **4.3.2 Meta-analysis**

We defined the primary outcome as the weighted (unstandardized) mean difference (WMD) between infarct sizes in the RIC and control groups. WMD was used as all data were presented in the same units and it gives a biologically relevant value. In each publication, we identified all independent comparisons of IS/AAR% in RIC versus control groups. Where a study made multiple comparisons to the same control group, the size of the control group was corrected for the number of comparisons made ( $n/\text{number of comparisons}$ ) [351]. The secondary outcome was the effect of 5 pre-defined experimental variables, which we considered most likely to impact on the efficacy of RIC, on WMD.

Comparisons were grouped according to their use of either remote ischaemic preconditioning (RIPreC), or remote ischaemic perconditioning and postconditioning (RIPerC/RIPostC). This is due to temporal differences in their application that, despite not necessarily occurring via different mechanistic pathways [232], have different clinical utility. Specifically, RIPerC/RIPostC are clinically applicable to STEMI, whereas RIPreC is not. Subsequent analysis was performed for each group separately.

For each independent comparison, we calculated the effect size as a raw difference in IS/AAR% means (the mean of the control groups minus the

mean of the experimental group) and the corresponding 95% confidence interval (CI). To account for anticipated heterogeneity, we pooled effect sizes using random-effects meta-analysis, which considers the within-study and between-study variability and weights each study accordingly. Heterogeneity was quantified using I<sup>2</sup> and T<sup>2</sup> statistics. Studies with missing data on any of the pre-defined experimental variables were excluded from the meta-analysis.

Subgroup analyses were performed using univariate meta-regressions to explore which experimental factors and quality indicators contribute to heterogeneity. Pre-defined experimental measures included in the analysis were as follows:

1. **Species:** Studies that used either mice or rats were grouped as 'small animals', and those using rabbits or pigs were grouped as 'large animals'.
2. **Cycle duration:** The reported duration of ischaemia applied to the limb in each cycle. In the analysis, studies were grouped as 5, 10 or 15 minutes of limb ischaemia.
3. **Number of cycles:** The number of times this ischaemic episode was applied in succession to the limb. Studies were grouped as using 1, 3 or 4 cycles of ischaemia.
4. **Number of limbs:** The RIC protocol reported by each study was either applied to one or both (i.e. bilateral) limbs. Conditioning by infra-renal aortic occlusion was classed as bilateral hindlimb ischaemia, whereas studies using supra-renal aortic occlusion were excluded.
5. **Oxygen:** Each study was given a binary score according to its reported use of supplementary oxygen in their ventilation protocol for the *in vivo* procedure. If the study did not mention whether supplementary oxygen was used, it was assumed the animal was ventilated with room air.

The percentage of between-study variance explained by variables of interest was assessed using the T<sup>2</sup> and adjusted R<sup>2</sup> statistics. The significance level was adjusted according to the number of comparisons using the Holm-Bonferroni method and results were considered significant when  $P < 0.01$  [147].

Sensitivity analysis was performed to assess the robustness of our findings by performing an additional analysis for both the primary and the secondary endpoints using the standardized mean difference (SMD; the mean of the control group minus the mean of the RIC group, divided by the pooled SD of the two groups). We performed a stratified meta-analysis by subgroup to validate the results obtained by meta-regression.

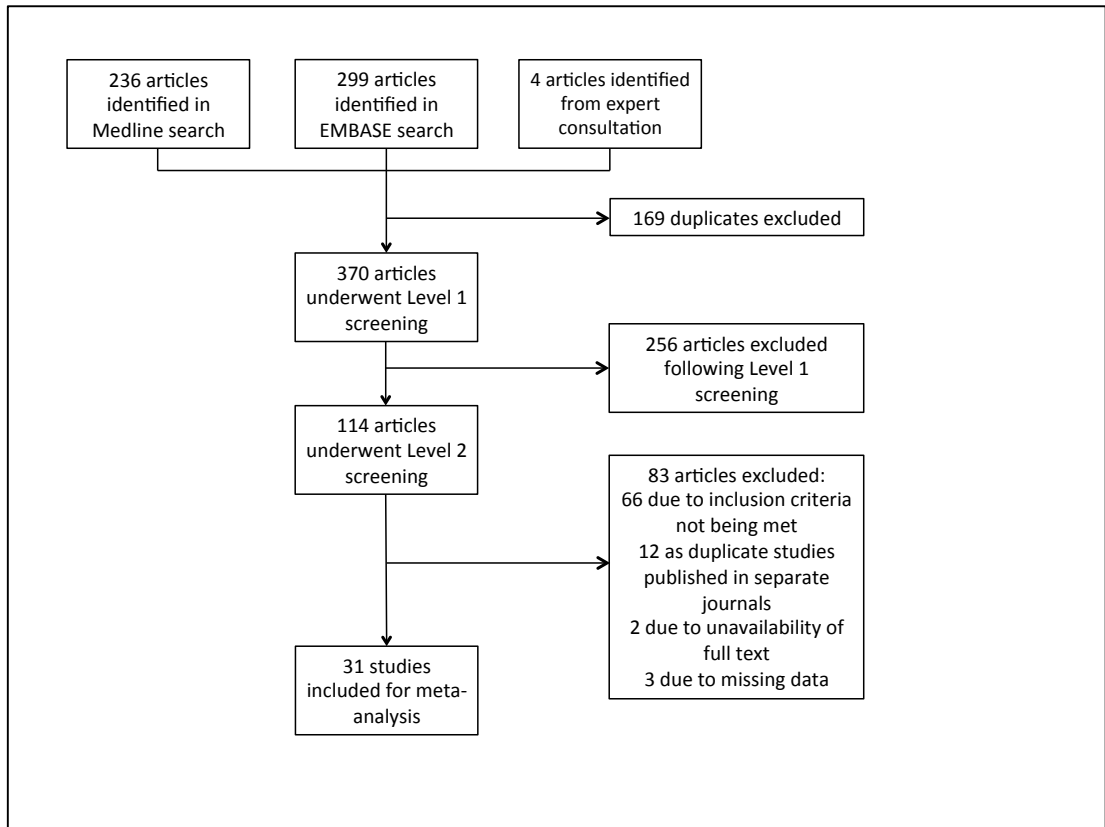
All analyses were pre-specified and performed using STATA/SE, version 13.1 (StataCorp, College Station, TX, USA); GraphPad Prism version 5.00 for Windows (CA, USA) and Adobe Illustrator CS6 (CA, USA) were used in the production of figures.

## **4.4 Results**

### **4.4.1 Study selection**

Our search returned 539 records, including 169 duplicate reports (consisting of reports returned by both Medline and Embase). 370 reports underwent title and abstract screening, which resulted in 256 exclusions. The remaining 114 reports were retrieved for detailed full text evaluation. 83 articles were excluded, 66 due to failing to meet the inclusion criteria, 12 were abstracts and 2 were not retrievable. Of the remaining 34 reports (studies), 3 were missing data on one or more important experimental variables that we were unable to retrieve by contacting the study authors, and were consequently excluded. The remaining 31 studies were included in the quantitative synthesis (**Fig 4-1**).





**Figure 4-1. Flow diagram demonstrating study exclusion during systematic review.**

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
[4]	Pig	M	25-30kg	Per	5	5	4	1	20	Clamp	N/A	40	LAD	120	NR	Y	Propofol, fentanyl	N	SEM	10	59.2	3.17	7
[9]	Pig	M/F	18-22kg	Post	5	5	4	1	20	Cuff	N/A	90	LAD	4320	R	Y	Ketamine	Heparin	SEM	12	48.4	5.2	12
[24]	Rat	M	Adult	Pre	15	N/A	1	2	30	Clamp	10	30	LAD	120	NR	N	Pentobarbitone	N	SEM	8	41	2	12
	Rat	M	Adult	Per	15	N/A	1	2	30	Clamp	N/A	30	LAD	120	NR	N	Pentobarbitone	N	SEM				10
	Rat	M	Adult	Per	15	N/A	1	2	30	Clamp	N/A	30	LAD	120	NR	N	Pentobarbitone	N	SEM				10
	Rat	M	Adult	Post	15	N/A	1	2	30	Clamp	N/A	30	LAD	120	NR	N	Pentobarbitone	N	SEM				10
[55]	Rat	M	Adult	Pre	5	5	4	1	20	Clamp	5	40	LAD	120	NR	N	Pentobarbitone	N	SEM	6	66.5	5.5	6
[111]	Rat	M	8-10 weeks	Per	10	10	1	1	10	Tourniquet	N/A	40	LAD	120	NR	N	Pentobarbitone	N	SEM	6	54.9	6.01	6
[125]	Pig	M	27-35kg	Pre	5	5	4	1	20	Clamp	5	60	LAD	180	NR	Y	Propofol, pancuronium, fentanyl	Heparin	SEM	5	48.8	4.2	5
	Pig	M	27-35kg	Per	5	5	4	1	20	Clamp	N/A	60	LAD	180	NR	Y	Propofol, pancuronium, fentanyl	Heparin	SEM				6
[137]	Rat	M	Adult	Pre	5	5	4	2	40	Cuff	10	35	LAD	120	NR	Y	Pentobarbitone	N	SD	10	76	14	10
[142]	Rat	M	8-10 weeks	Pre	10	10	1	1	10	Clamp	10	40	LAD	120	NR	N	Pentobarbitone	N	SEM	9	64.9	2.6	11
[159]	Rat	M	288+/-9kg	Per	10	10	1	1	10	Tourniquet	N/A	40	LAD	120	NR	N	Pentobarbitone	N	SEM	6	54.6	4.7	6
[168]	Rat	M	Adult	Pre	5	5	4	1	20	Clamp	5	40	LAD	120	NR	N	Pentobarbitone	N	SEM	7	65.3	2.9	6
[169]	Mouse	M	N/R	Pre	5	5	4	1	20	Clamp	5	30	LAD	120	NR	N	Pentobarbitone	N	SEM	9	40.6	3.6	6
	Rat	M	2-3 months	Pre	5	5	4	1	20	Clamp	5	40	LAD	120	NR	N	Pentobarbitone	N	SEM	7	65.1	2.7	6
[185]	Pig	N/R	15kg	Pre	5	5	4	1	20	Tourniquet	5	40	LAD	120	NR	Y	Midazolam, pentobarbitone	Heparin	SEM	8	53	8	9
[189]	Rat	M	N/R	Per	15	N/A	1	2	30	Clamp	N/A	30	LAD	120	NR	N	Pentobarbitone	N	SEM	10	69	2	10
	Rat	M	N/R	Per	15	N/A	1	2	30	Clamp	N/A	30	LAD	120	NR	N	Pentobarbitone	N	SEM	6	68.6	0.8	6
[217]	Rabbit	M/F	2.5-3kg	Per	5	1	1	1	5	Clamp	N/A	30	LAD	180	NR	Y	Pentobarbitone	Heparin	SD	10	31.5	1.3	10
[223]	Mouse	M	10-12 weeks	Pre	5	5	3	1	15	Clamp	5	30	LAD	120	NR	Y	Ketamine, xylazine, atropine	N	SEM	10	56.7	3.2	9
[234]	Rat	M	280-300g	Pre	5	5	1	1	5	Clamp	5	30	LAD	120	NR	N	Pentobarbitone	N	SD	6	54.7	6	6
	Rat	M	280-300g	Pre	5	5	3	1	15	Clamp	5	30	LAD	120	NR	N	Pentobarbitone	N	SD				6
[243]	Rat	M	Adult	Pre	15	10	1	2	30	Clamp	10	30	LAD	120	NR	N	Pentobarbitone	N	SEM	7	54.6	3.1	8
[308]	Rat	F	200-250g	Per	5	5	4	2	40	Clamp	N/A	45	LAD	120	NR	N	Ketamine, xylazine	N	SEM	22	48.7	3.4	22
[311]	Pig	N/R	20kg	Per	5	5	4	1	20	Tourniquet	N/A	40	LAD	120	NR	Y	Midazolam, pentobarbitone	Heparin	SEM	10	60	5	10

[318]	Rat	M	Adult	Pre	15	10	1	2	30	Clamp	10	30	LAD	120	NR	N	Urethane	N	SEM	6	55.5	3.1	6
[336]	Rat	M	8-10 weeks	Per	10	10	1	1	10	Tourniquet	N/A	40	LAD	120	NR	N	Pentobarbitone	N	SEM	6	54.9	6.5	6
[287]	Mouse	M	12±3 weeks	Pre	5	5	4	1	20	Cuff	5	30	LAD	1440	R	Y	Ketamine, xylazine	N	SD	5	37	4	5
[364]	Rat	M	250-280g	Per	5	5	4	1	20	Tourniquet	N/A	45	LAD	4320	R	N	Pentobarbitone	Heparin	SD	8	50.5	4.1	8
[367]	Rat	M	250-350g	Pre	15	0	1	2	30	Clamp	0	30	LAD	120	NR	Y	Pentobarbitone	N	SEM	6	62	5	6
	Rat	M	250-350g	Pre	5	10	1	2	10	Clamp	10	30	LAD	120	NR	Y	Pentobarbitone	N	SEM				6
	Rat	M	250-350g	Pre	10	10	1	2	20	Clamp	10	30	LAD	120	NR	Y	Pentobarbitone	N	SEM				6
	Rat	M	250-350g	Pre	15	10	1	2	30	Clamp	10	30	LAD	120	NR	Y	Pentobarbitone	N	SEM				8
[373]	Rat	M	300±25g	Pre	5	5	3	1	15	Clamp	5	30	LAD	120	NR	N	Pentobarbitone	N	SD	6	52.8	5.9	6
[379]	Rat	M	8 week	Per	5	5	4	1	20	Cuff	N/A	45	LAD	120	NR	N	Pentobarbitone	Heparin	SD	8	48.9	6.66	8
[381]	Rat	M	8-9 weeks	Per	10	N/A	1	2	20	Tourniquet	N/A	30	LAD	180	NR	Y	Pentobarbitone	N	SD	9	60	3	9
[395]	Rat	M	250-300g	Per	5	5	3	1	15	Clamp	N/A	45	LAD	180	NR	N	Pentobarbitone	Heparin	SEM	12	55.8	2.2	12
[401]	Rat	M	8 weeks	Per	10	N/A	1	2	20	Tourniquet	N/A	30	LAD	120	NR	Y	Pentobarbitone	N	SD	20	71.6	8.7	20
[403]	Rat	M	230-260g	Pre	5	5	3	1	15	Clamp	5	30	LAD	120	NR	Y	Chloral hydrate	N	SD	6	51	6	6
[407]	Rat	M	Adult	Pre	5	5	3	2	30	Tourniquet	5	30	LAD	180	NR	N	Chloral hydrate	Heparin	SEM	12	34.7	5.9	12
	Rat	M	Adult	Per	5	5	3	2	30	Tourniquet	N/A	30	LAD	180	NR	N	Chloral hydrate	Heparin	SEM				12
	Rat	M	Adult	Post	5	5	3	2	30	Tourniquet	N/A	30	LAD	180	NR	N	Chloral hydrate	Heparin	SEM				12

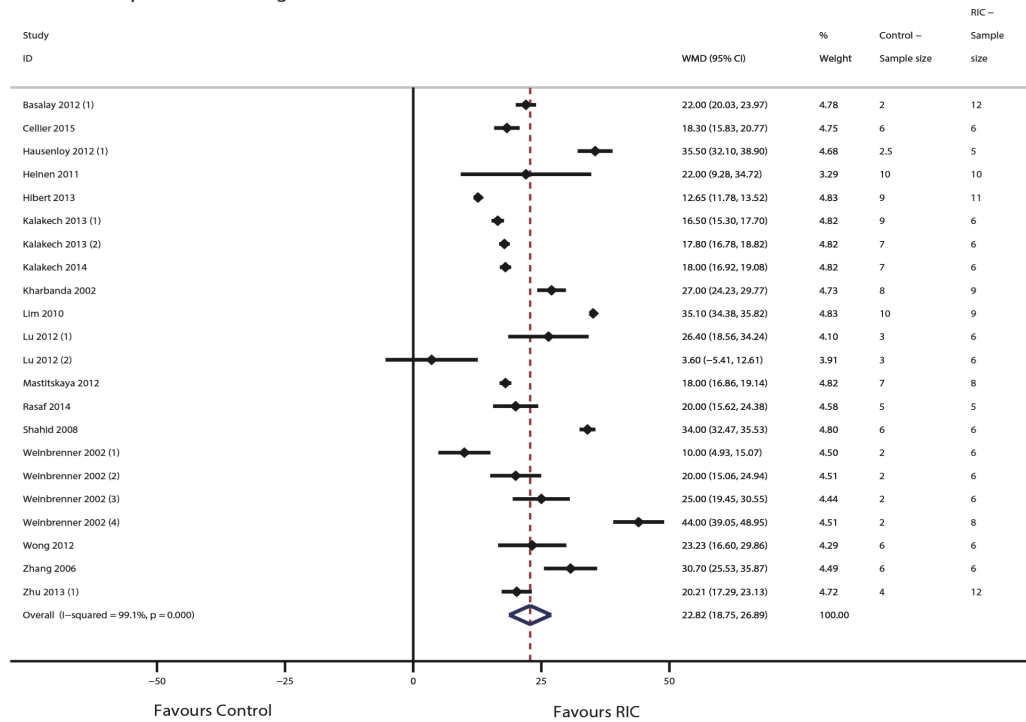
**Table 4-4. Main characteristics of included studies.** The main characteristics included: (1) Study reference; (2) Species; (3) Gender; (4) Age or weight; (5) Conditioning protocol (pre-, per- or post-conditioning); (6) RIC cycle duration (min); (7) RIC reperfusion duration (min); (8) Number of cycles; (9) Number of limbs; (10) Total RIC ischaemia duration (min); (11) RIC occlusion technique; (12) Time between RIC and index ischaemia (min, preconditioning only); (13) Index ischaemia duration (min); (14) Coronary artery occluded; (15) Reperfusion duration (min); (16) Recovery or non-recovery; (17) Supplementary oxygen; (18) Induction anaesthetic; (19) Anticoagulants; (20) Measure of variance; (21) Control group sample size; (22) Control group mean infarct size (IS/AAR%); (23) Control group variance (IS/AAR%); (24) Conditioning group sample size; (25) Conditioning group mean infarct size (IS/AAR%); and (26) Conditioning group variance (IS/AAR%). N/R, not recorded; N/A, not applicable; LAD, left anterior descending; R, recovery; NR, non-recovery; SEM, standard error of the mean; SD, standard deviation.

#### 4.4.2 Meta-analysis

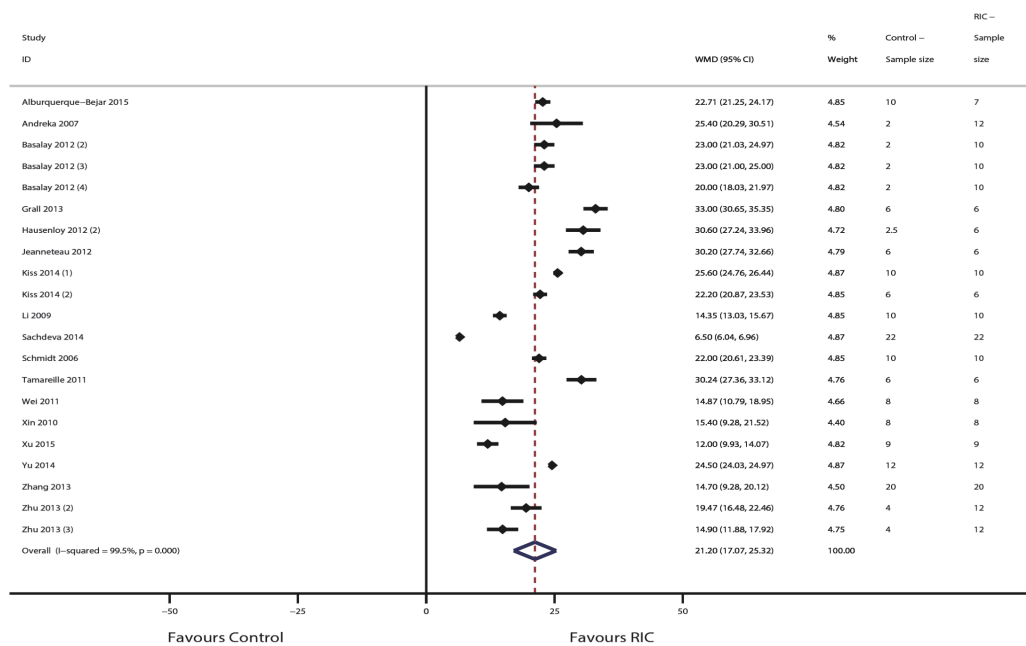
From the 31 included reports, we extracted data on 43 controlled comparisons of RIC in models of myocardial IRI. These were split into 22 comparisons investigating RIPreC, and 21 comparisons investigating RIPerC/RIPostC. In total, our analysis includes data from 280 control animals and 373 animals undergoing RIC. In the RIPreC group, conditioning reduced IS/AAR% by 22.8% (95% CI 18.8-26.9%) when compared to untreated controls ( $P<0.001$ ;  $n=22$  comparisons, **Figure 3-2 A**). Significant heterogeneity was observed ( $T^2=89.2$  and  $I^2=99.1\%$ ;  $P<0.001$ ). In the RIPreC/RIPostC group, conditioning reduced IS/AAR% by 22.2% (95% CI 17.1-25.3%) when compared to untreated controls ( $P<0.001$ ;  $n=21$  comparisons, **Figure 3-2 B**). Again, significant heterogeneity was observed ( $T^2=90.9$  and  $I^2=99.5\%$ ;  $P<0.001$ ).

We investigated potential experimental sources of the observed heterogeneity using meta-regression analysis with IS/AAR% as the dependent variable, and did not find any significant associations with efficacy of RIC (**Figure 3-3**).

## A. Remote preconditioning

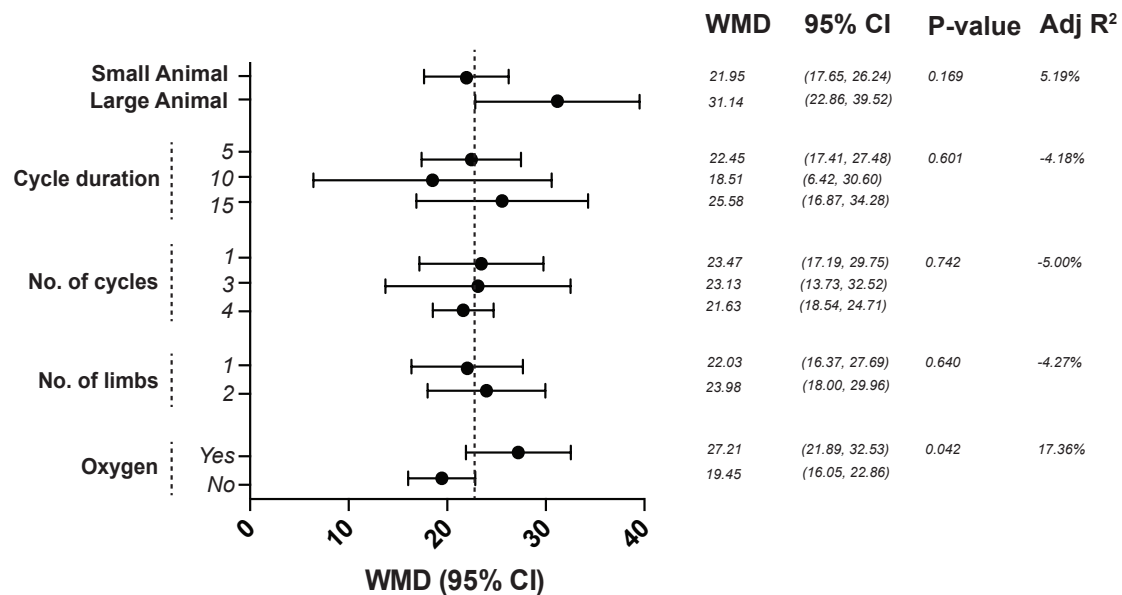


## B. Remote preconditioning and postconditioning

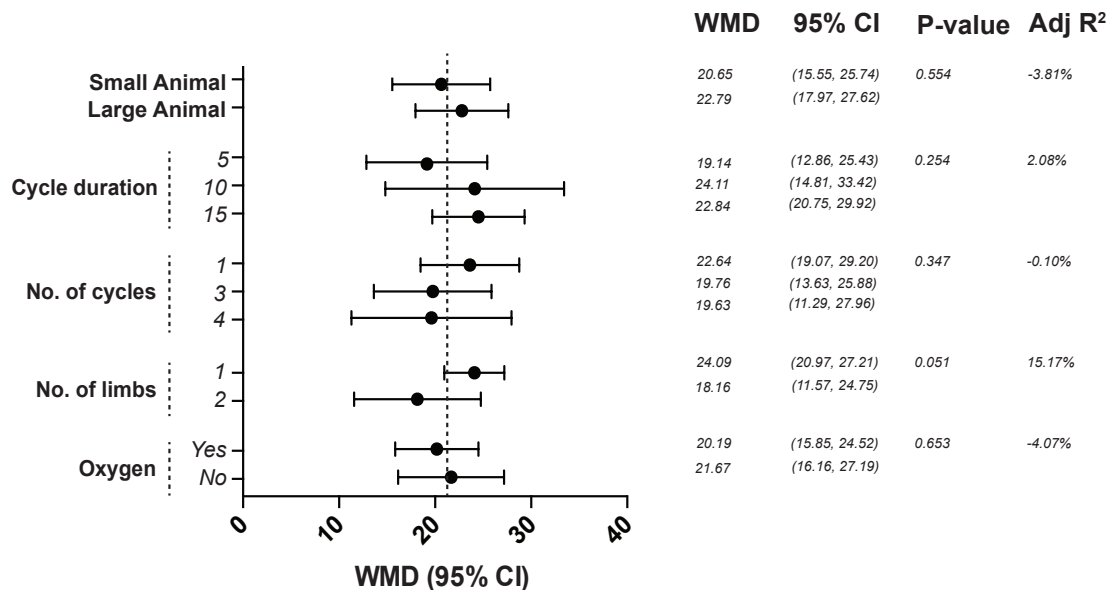


**Figure 4-2. Forest plots detailing the weighted mean difference for each included study. Forest plots of the effect of A) RIPreC and B) RIPerC/RIPostC on IS/AAR%, pooled using random-effects meta-analysis. 22 and 21, respectively, controlled comparisons were included, amounting to data from 280 control animals and 373 animals undergoing RIC. Red dotted line indicates the average effect size (RIPreC = 22.8% and RIPerC/RIPostC = 22.2%).**

## A Preconditioning Studies



## B Per-/Post-conditioning Studies



**Figure 4-3. Impact of experimental factors on the efficacy of A) RIPreC and B) RIPerC/RIPostC.**

Weighted mean difference (WMD) and the corresponding 95% CI for each variable were obtained by subgroup stratification. However, the reported P-value was obtained by meta-regression to reduce false-positive findings. Studies that used either mice or rats were grouped as 'small animals', and those using rabbits or pigs were grouped as 'large animals'. A P value of <0.01 was considered significant.

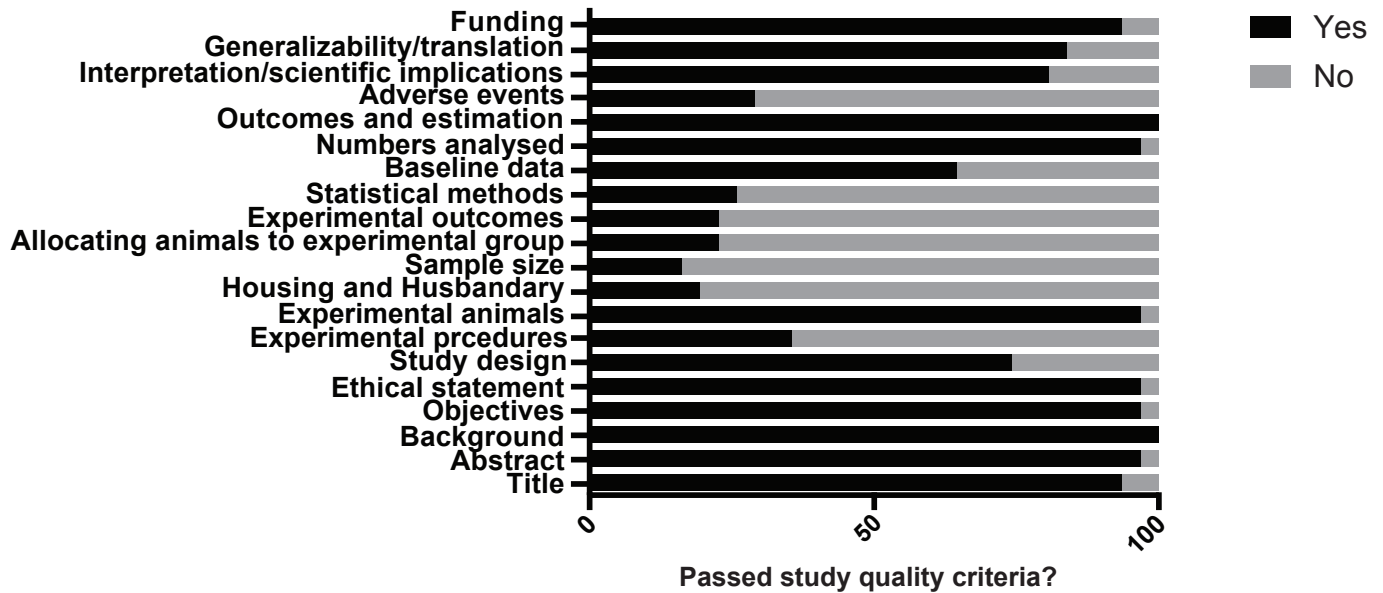
#### 4.4.3 Risk of bias

Reports achieved a median ARRIVE guidelines score of 14 (interquartile range 12-14) out of 20 and a median 12-item quality score of 7 (interquartile range 6-8; Figure 4-4). A full breakdown of the scores is given in the Supplementary material, *Tables S7-8*. Meta-regression indicated that study quality according to either the ARRIVE guidelines score or to a 12-item quality score was not associated with the overall effect ( $P=0.317$  and  $P=0.846$ , respectively).

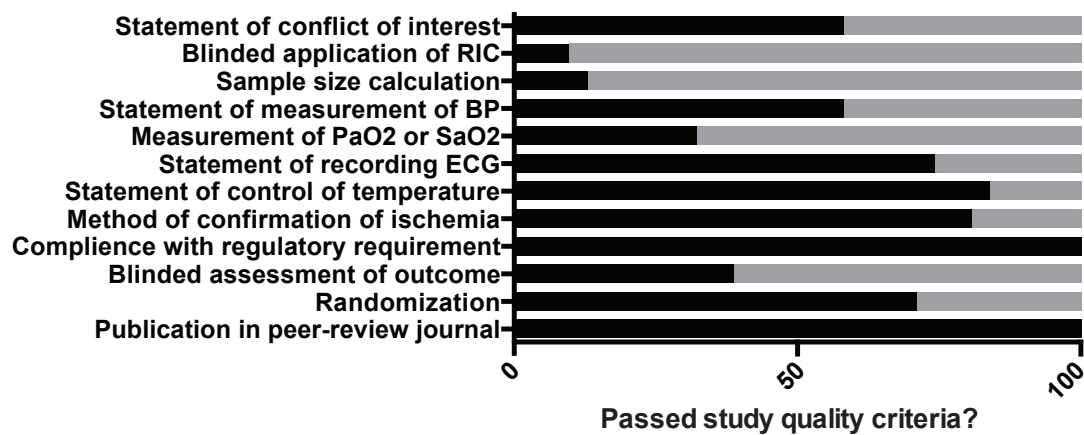
Overall, studies performed particularly poorly in several important areas of experimental design. For example, with respect to the ARRIVE guidelines only 16% reported sample size calculation, 23% reported randomization of animals to experimental groups, 23% defined the primary experimental outcome and 29% reported detail of adverse events during experiments (Figure 4-4 A). Regarding the 12-item quality score, only 32% of studies reported measurement of blood oxygen saturation, 13% reported measurement of blood pressure during the *in vivo* protocol and 10% performed blinded application of the conditioning protocol (Figure 4-4 B). Notably, only 38% of studies performed a blinded assessment of outcome.

The impact of publication bias on the overall effect was assessed by visual analysis of the funnel plot, which suggested that small and negative studies might be under-represented (Figure 4-5). However, in Egger's regression test the null-hypothesis of no small-study effect was not rejected at  $P=0.216$  (estimated bias coefficient  $3.75 \pm 2.98$  SE).

## A. ARRIVE guidelines



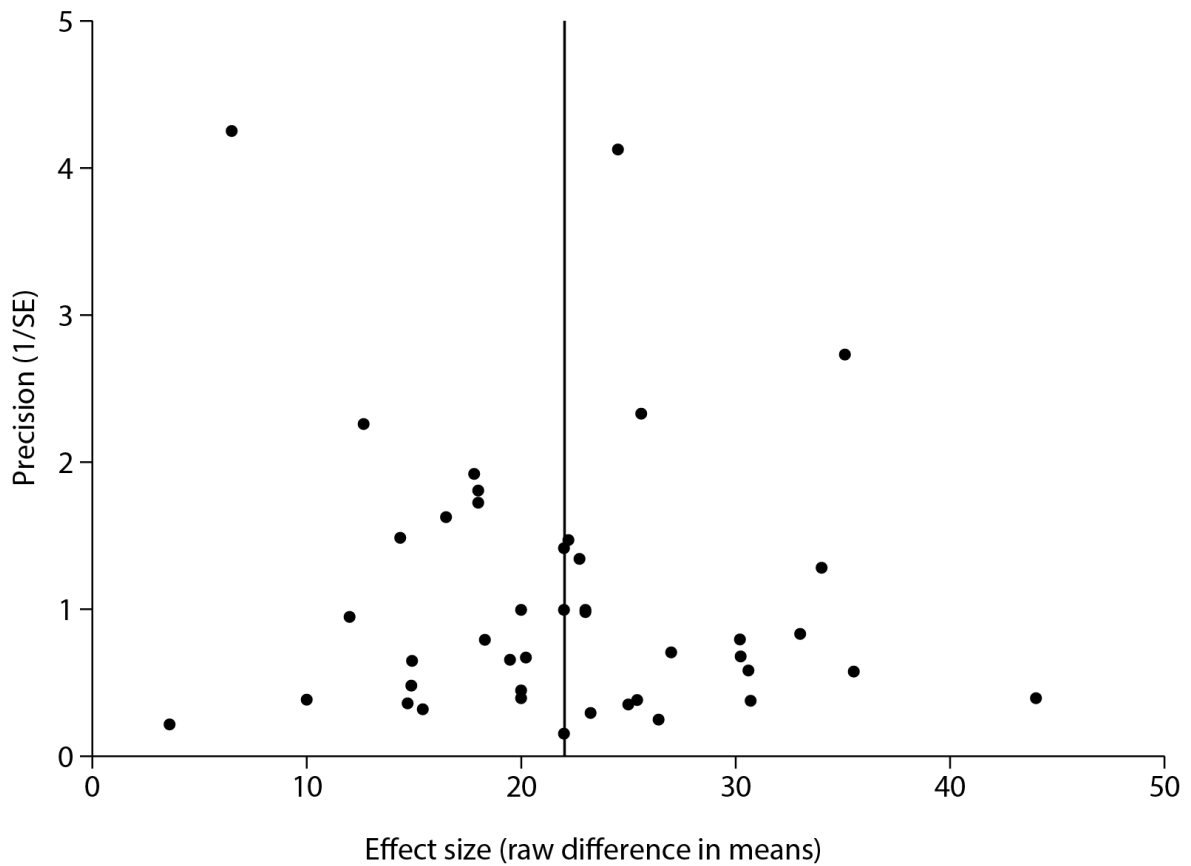
## B. Study quality score



**Figure 4-4. Reporting of study quality indicators.**

Study quality was assessed using the ARRIVE guidelines on reporting *in vivo* experiments (A) and a 12-item quality score (B). Values are expressed as the percentage of studies reporting each quality indicator.





**Figure 4-5. Assessment of publication bias.**

*A funnel plot comparing treatment effect to a measure of study size (precision of the effect estimate). The vertical line represents the mean effect size. This plot was assessed visually, with further analysis of publication bias performed using Egger's regression test.*

#### **4.4.4 Sensitivity analysis**

When re-running our analysis using the SMD, all results were similar to those found using the WMD. We found a highly significant ( $P < 0.001$ ) overall effect of RIPreC (SMD of 11.06; 95% CI 8.52-13.60), as well as a similar level of heterogeneity ( $I^2 = 91.1\%$ ). None of the experimental variables were significant after correction by multiple comparison. For RIPerC/RIPostC, all results using the SMD were likewise similar to those found using the WMD. We found a highly significant ( $P < 0.001$ ) overall effect (SMD of 13.14; 95% CI 10.51-15.77) and similar heterogeneity ( $I^2 = 93.3\%$ ). None of the experimental variables were significant after correction by multiple comparison.

#### **4.5 Discussion**

Our major finding is that both RIPreC and RIPerC/RIPostC have a large beneficial effect of both on infarct size. This finding is based on a comprehensive systematic review, including over 650 animals. However, there were relatively few pre-clinical studies investigating RIC and even fewer systematically characterising the protocol, which limited our analysis.

Furthermore, we found inconsistency in the design of pre-clinical RIC studies with very few that were randomized effectively, included both male and female animals, and were double blinded with respect to procedure and outcome, which highlights the need for further well-designed, pre-clinical studies of RIC.

These are important findings in the context of recent pessimism regarding RIC as a genuine cardioprotective phenomenon [309, 400]. Clinical trials investigating the efficacy of RIC in myocardial IRI have had mixed results [41, 122], which has been attributed to clinical variables, including propofol administration [198]. However, each of the factors described above may influence outcome through lack of generalizability or unconscious bias [211], and therefore indirectly impact upon attempts to translate RIC to humans in clinical trials [99].

#### 4.5.1 Determinants of RIC

Interestingly, we found high levels of heterogeneity between studies. To investigate whether study protocol could account for the observed heterogeneity, and to elucidate the determinants of efficacy of RIC, we assessed the impact of experimental variables on effect size using a meta-regression analysis. This approach has been successfully applied to several promising pre-clinical interventions to date. For example, Lim *et al.* were able to demonstrate that ciclosporin was not effective at limiting myocardial infarct size in pig models of IRI, compared to small animal models [224]. This finding might be important in the context of a subsequent neutral clinical study of ciclosporin before reperfusion in patients with STEMI [69]. Therefore, finding the parameters responsible for heterogeneity can guide pre-clinical and clinical study design.

However, none of the tested experimental variables, which included species, cycle duration, number of cycles, number of conditioned limbs and the use of supplementary oxygen, were associated with effect size. In a recent analysis of pre-clinical studies of local ischaemic preconditioning, which included limited analysis of RIC, species (rodent vs. non-rodents) accounted for a substantial amount of the observed heterogeneity [368]. Although we found no significant association between animal size (rodents vs. non-rodents), there was a pattern of increased efficacy of RIPreC in large animals.

Wever *et al.* also describe no association between effect size and the number, timing and duration of cycles, which is reflected in the present study [368]. Specifically, we found 1, 3 and 4 cycles to be equally effective. Conversely, in one of the few neutral comparisons in our analysis, Lu *et al.* utilised a protocol consisting of 1 cycle of 5 min RIPreC [234]. Interestingly, this amounts to the lowest total ischaemic 'dose' (a function of cycle number and duration) of any study of RIPreC. Amongst the RIPerC/RIPostC studies, only Li *et al.* applied 1 cycle of 5 min limb ischaemia and reported a relatively modest, albeit significant, effect (WMD 14.35%, 95% CI 13.03-15.67%) [217]. Others, including Mastitskaya *et al.*, have applied a single cycle of longer

duration and achieved greater protection [243]. It is therefore plausible that ischaemic 'dose' or burden, rather than cycle number or duration alone, is the dependent variable, but very few studies have examined this systematically *in vivo*. An exploratory analysis of the present data demonstrated no such association (data not shown), however this was limited by a narrow distribution of total ischaemic times in our dataset. Interestingly, a dose-dependent effect of RPreC has been observed, with greater cardioprotection after 10 min or 15 min compared to 5 min continuous infra-renal aortic occlusion [365]. In a study comparing bilateral and unilateral RPreC in protection against renal ischaemia-reperfusion injury, bilateral was found to be more effective [369]. Taken together, this may suggest that total ischaemic dose is important, but this needs testing in specifically designed experiments.

Furthermore, there may be an upper limit to what is an effective ischaemic dose. A recent characterisation of the RIC protocol *ex vivo* reported that 4 and 6, but not 8, cycles of RIC were protective in mice [166]. Similarly, although 5 min or 10 min limb ischaemia protected against subsequent liver injury, 30 min and 60 min actually increased injury [359]. This issue is worthy of further attention, particularly using a more realistic setting of aged animals with co-morbidities. This may suggest a therapeutic window for RIC but there is very limited pre-clinical evidence, in contrast to direct ischaemic conditioning where the number of cycles are demonstrably important [368], and it is clear that detailed characterisation of study protocol *in vivo* is urgently necessary in order to answer these questions.

After a period of limb ischaemia, it has been assumed that reperfusion is necessary, either to wash out the putative humoral factor, or because reperfusion-induced ROS may be necessary to activate signalling pathways. Additionally, some studies have suggested that reactive hyperaemia is an important factor in the response [287]. However it is worth bearing in mind that the original description in rabbits, where RPreC was achieved by 55-65% stenosis of the femoral artery in combination with rapid electrical stimulation of the gastrocnemius muscle for 30 min, did not actually involve

reperfusion [35]. This appears to support the hypothesis that multiple mechanisms are involved [223, 278].

Similarly, our analysis supports the finding of Wever *et al.* of no association with the number of limbs conditioned [368]. Johnsen *et al.* further reported that 2 and 5 min, in contrast to 10 min, cycles were beneficial in mice [166]. This remains consistent with the concept of a therapeutic window of RIC but there is a paucity of in vivo experimental data relating to the precise RIC protocol. Our analysis demonstrated 10 min cycles to be equally effective as 5 min cycles, albeit only in larger species as no studies in our meta-regression used 10 min cycles in mice. We also noticed a pattern of reduced efficacy in RIPreC/RIPostC studies using 2 limbs instead of one, which may exceed the therapeutic window. However, this finding was not statistically significant and should only be considered hypothesis-generating.

The use of supplementary oxygen in acute myocardial infarction is controversial [332], but the role of supplementary oxygen in RIC has not, to our knowledge, been investigated. It could be hypothesised that RIC is driven by cellular hypoxia in the conditioned limb which, at least in part, might be alleviated by ventilation with supplementary oxygen. In addition, excessive blood oxygen tension could increase production of reactive oxygen species, which has been shown to increase sympathetic drive [52]. This could impair the efficacy of RIC given the proposed importance of the vagus nerve [243, 278]. However, despite finding the conditioning of animals with and without oxygen to be equally effective, there was a pattern of increased efficacy in animals ventilated with supplementary oxygen in the RIPreC comparisons. This has not been specifically investigated but our results would suggest further investigation is warranted.

Furthermore, there is evidence that the degree of cardioprotection conferred by RIC is proportional to the duration of index ischaemia. Specifically, Kleinbongard *et al.* demonstrated longer ischaemic times to be associated with a greater efficacy of RIC, albeit in a study of patients undergoing CABG, which may be related to a greater target for protection [191]. In an

exploratory analysis, we found no statistical effect of index ischaemic time (Tables 4-5 & 4-6); however, there was a pattern of reduced efficacy of RIC at longer ischaemic durations in RIPreC but not RIPerC/RIPostC. This might suggest an important role for the timing of intervention, but should be interpreted with caution in view of the limited number of comparisons available after stratification according to study protocol.

Experimental factor	WMD	(95% CI)	% Weight	P-value	Adj R-squared
Duration of isch index				0.180	6.41%
30	23.42	(18.26, 28.58)	75.16		
35	22.0	(9.28, 34.72)	3.63		
40	16.63	(13.53, 19.73)	21.20		
45	-	-	-		

**Table 4-5. Meta-analysis comparing the effect of index ischaemia duration on WMD in studies investigating remote PREconditioning. *N=20 comparisons; the 35 min group includes only 1 comparison.***

Experimental factor	WMD	(95% CI)	% Weight	P-value	Adj R-squared
Duration of isch index				0.997	-3.09%
30	19.63	(16.54, 22.60)	56.44		
35	-	-	-		
40	31.24	(29.35, 33.13)	18.84		
45	15.32	(2.89, 27.75)	24.73		

**Table 4-6. Meta-analysis comparing the effect of index ischaemia on WMD in studies investigating remote PERconditioning and POSTconditioning. *N*=16 comparisons.**

There were several experimental variables that were of considerable interest but that lacked sufficient power for statistical analysis. For example, it has been suggested that propofol interferes with the development of RIC [198], and it has been implicated as a potential reason for the apparent lack of translation of RIC [140, 141]. Interestingly, we observed studies where propofol (together with either opioid analgesia  $\pm$  pancuronium) was administered to have effect sizes above the mean [4, 125].

Furthermore, studies reporting the use of mixed gender experimental groups reported apparently smaller effect sizes, and one of the few neutral studies was performed only in female animals [308]. Gender can potentially impact upon ischaemia-reperfusion injury possibly due to the cardioprotection conferred by oestrogen [253], and potential temporal variability in cardioprotection as a result of the oestrous cycle of female rats [37]. However, investigation of the role of gender is conspicuously absent from pre-clinical studies of RIC and these qualitative data require further testing in formal, well-designed studies.

An important result of the present study is the need for more pre-clinical characterisation of RIC, which as well as the aforementioned variables should include the potential role of occlusion technique (arterial clamping vs. external compression), which may be important due to the possible involvement of limb collaterals [244], the putative effect of shear stress in RIC [287], and the role of pre-treatment with heparin, which has been reported to be cardioprotective in the context of IRI [36, 110, 199]. Likewise, it has been hypothesised that the interval between the conditioning stimulus and the onset of index ischaemia might be an important determinant of the efficacy of remote preconditioning [99].

A further consideration is the impact of co-morbidities and co-mediations on the efficacy of RIC. The animal models used in pre-clinical studies frequently do not reflect the complex risk factor, co-morbidity and pharmaceutical profile of humans with cardiovascular disease [124, 282], which may impede the development of RIC. For example, Jensen *et al.* treated isolated perfused rabbit hearts subjected to IRI with plasma dialysate from diabetic patients treated with RIC [164]. They were unable to confer cardioprotection using dialysate from patients with peripheral neuropathy compared to non-diabetic patients and diabetic patients without neuropathy. Baranyai *et al.* similarly found that acute hyperglycaemia abrogated the beneficial effect of RPerC in Wistar rats [22]. However, despite considerable investigation of these features in local ischaemic preconditioning (reviewed by Ferdinandy *et al.* [92]), there is little experimental data relating to these factors in the context of RIC. Indeed, animals with co-morbidities were specifically excluded from the present analysis due to the small number of studies available. Nonetheless, co-morbidities should be considered when investigating the optimum protocol for the delivery of RIC.

#### **4.5.2 Risk of bias**

Poor methodological quality and publication bias can result in over-estimation of effect size [316, 374, 375]. In turn, this can engender enthusiasm about the benefit of a treatment where, in fact, none exists. It is therefore essential to examine the impact of study quality on size of effect, to which end we found no statistical relationship using meta-regression.

However, we made some interesting observations. Aspects of the report relating to the experimental procedure, including control of temperature and recording of the ECG, were generally well reported. However, there was generally poor observation of the ARRIVE guidelines, particularly in relation to reporting of sample size calculations, randomisation, blinding and adverse events, which can result in selective exclusion. For example, in an interesting meta-analysis of systematic reviews, Hirst *et al.* reported that failure to randomise significantly increased effect size [145]. Furthermore, appropriate



monitoring of experimental animals, including recording of blood oxygen saturation and blood pressure, were poorly reported. These facets are clearly essential to ensure good quality research and were a central tenet of a recent position paper on improving the pre-clinical assessment of novel cardioprotective therapies [211]. In some cases these omissions will represent inadequate reporting but in others it is likely that these crucial elements of study design were not performed. We elected not to analyse each quality criterion independently due to insufficient reporting and to avoid false positive findings due to multiple comparisons. However, poor adherence to certain quality criteria may account for the heterogeneity observed in this meta-analysis.

Finally, our assessment of publication bias by visual analysis of the funnel plot suggested that small, neutral studies may be under-represented; however, this did not statistically impact on the overall effect size, which is reassuring.

#### **4.5.3 Limitations**

The validity of this meta-analysis is contingent upon the quality of reporting of the included studies. Unpublished studies and those with missing data could not be included in the meta-analysis, and others did not meet important quality criteria including poor information regarding statistical analysis and blinding. However, the absence of a statistical impact from study quality or publication bias is reassuring in this regard. We were limited by being unable to consider manuscripts not available in English and we acknowledge that we did not carry out a systematic literature review to determine which experimental variables to include in our analyses, which might be subject to selection bias. A relatively small number of studies were included in the meta-analysis, thereby limiting the power of the study, which was further affected by multiple comparisons within individual studies; however, we elected to include all comparisons to avoid selection bias. Meta-regression is inherently limited; however, to ensure this was as robust as possible we performed a stratified meta-analysis by subgroup that yielded similar results,

including a highly significant overall effect of RIC, significant heterogeneity, and no effect of any of the experimental variables we included in our model.

#### **4.5.4 Recommendations and Conclusions**

This systematic review and meta-analysis of pre-clinical *in vivo* studies of myocardial IRI demonstrates a significant and highly reproducible beneficial effect of RIC. This effect was highly heterogeneous, a finding that may be due to un-measurable, multifactorial differences between individual experimenters and laboratories. Importantly, however, *in vivo* studies to date suggest the optimal RIC stimulus has not yet been identified. There was a paucity of pre-clinical characterisation of the RIC protocol and poor reporting of quality indicators. This is important not so the protocol can be translated to humans, but in order to understand the key parameters and/or markers that will facilitate optimization of the protocol in humans.

There has been a great deal of debate regarding neutral clinical studies of RIC [99, 309]. However, before we try to understand these failings and design future studies it is essential to fully describe RIC in pre-clinical experiments. At present, studies variably (and apparently randomly) apply the intervention to one or two limbs, for varying periods of time, with a variable number of cycles and with inconsistent timing with respect to the injurious ischaemic episode. To this end, we have identified a need for more, well-performed studies with a focus on characterisation rather than detailed elucidation of mechanisms. In particular, these should concentrate on investigating the potential impact of gender and the number, timing and duration of cycles on the efficacy of RIC. These aims would be greatly aided by the identification of a biomarker or a critical physiological parameter(s) that correlates with protection. More studies investigating the potential benefit of RIC in larger species are required before translation to humans and we highlight the potential role of supplementary oxygen as particularly interesting for exploration. Finally, future research should focus on investigating other potential reasons for neutral clinical studies of RIC, including co-morbidities and adjunctive therapies.

## Chapter 5 CO-DEPENDENCE OF THE NEURAL AND HUMORAL PATHWAYS IN THE MECHANISM OF REMOTE ISCHAEMIC CONDITIONING

Some of the work presented in this chapter has been recently published. Chapter 10 contains a copy of the published manuscript.

***Pickard JMJ, Davidson SM, Hausenloy DJ, Yellon DM (2016) Co-dependence of the neural and humoral pathways in the mechanism of remote ischaemic conditioning. Basic Res Cardiol 111:50. doi: 10.1007/s00395-016-0568-z***

### 5.1 Introduction

Remote ischaemic conditioning was first discovered in 1993, where brief ischaemia to the circumflex coronary bed induced protection from a subsequent left anterior descending coronary artery infarction [283]. This extension of classical ischaemic conditioning was a paradigm shift, and revealed for the first time that one vascular bed can communicate with others to protect them from ischaemia-reperfusion injury. Soon, other groups expanded the paradigm by looking at more remote areas from the heart. Gho et al demonstrated that 15min occlusion of the mesenteric artery was sufficient to induce cardioprotection [102]. A number of subsequent studies added to these data, with brief renal artery and hindlimb/skeletal muscle ischaemia both sufficient to protect the myocardium [35, 222, 270]. Indeed, it seems there is ability within mammals for the vasculature to communicate throughout the body and increase the resistance to ischaemic injury.

One of the key research interests following these data was in determining the pathway by which the cardioprotection is communicated from the conditioned limb to the myocardium. Perhaps the most logical pathway is via release of one or more humoral factors into the blood stream, which then move from the conditioned organ to the heart and protect. This theory was supported by the observation that reperfusion of the conditioned organ was necessary to induce the remote cardioprotection [102, 366]. Moreover, several studies

demonstrated that protection could be transferred from one isolated heart to the next. Dickson et al collected coronary effluent from an isolated rabbit heart following exposure to classical ischaemic preconditioning, which was able to protect a naïve isolated rabbit heart when perfused prior to ischaemia reperfusion injury [75] (model described in section 3.4). Moreover, the same group demonstrated that protection could also be transferred via transfusion of whole blood from *in vivo* rabbits, who had been preconditioned with brief renal artery ischaemia, to a naïve acceptor rabbit [77].

There was soon an added layer of complexity to the paradigm, with several publications proposing a neural pathway for RIC communication. Gho et al first demonstrated that the ganglion blocker, hexamethonium, abrogated the cardioprotection offered by brief (10min) mesenteric artery occlusion. In addition, remote conditioning via skeletal muscle ischaemia was also sensitive to ganglion blockade in human subjects. Subsequent studies corroborated these data, with Ding et al demonstrating increased firing of renal nerve afferents following brief renal artery occlusion. This was abrogated by pretreatment with the adenosine receptor antagonist 8-(p-sulphophenyl)theophylline (8-SPT). This suggested a local release of adenosine in the conditioned organ, which triggers RIC via activation of sensory afferent nerves. Indeed, an elegant study by Liem et al confirmed these data, where intra-mesenteric artery infusion of adenosine (10ug/min) was sufficient to induce cardioprotection. This adenosine-mediated remote cardioprotection was also sensitive to the ganglion blocker hexamethonium. Infusion of the same concentration of adenosine into the portal vein was not cardioprotective. Moreover, the same cardioprotection could be achieved by intra-mesenteric artery injection of bradykinin and calcitonin gene related peptide. These data above suggest that remote conditioning is triggered via release of small autocoids in the conditioned organ, which activates sensory afferent nerves. These sensory nerves carry the cardioprotective message away from the conditioned limb towards the heart. Finally, it is of interest that the triggers for RIC and classical IPC are similar. This is investigated in more detail in Chapter 7.

The neural and humoral pathways of RIC were initially thought of as mechanistically distinct. Indeed, when the mesenteric artery occlusion was increased to 25 min, hexamethonium no longer appeared to block the ensuing cardioprotection. This suggested a humoral component of RIC that existed outside of any neuronal dependency. In fact, the current literature indicates that both pathways are critically dependent on one another, and the communication of RIC occurs via a neuro-humoral process. A key study from Steensrud et al demonstrated that a plasma dialysate prepared from rabbits that had received limb RIC, could protect a naïve Langendorff rabbit hearts from ischaemia-reperfusion injury. When the limb RIC was preceded with femoral nerve section, the resulting dialysate no longer protected the naïve heart. Moreover, intra-femoral artery adenosine in rabbits produced a plasma dialysate that was cardioprotective. This effect was also abrogated by prior femoral nerve section, and intra-femoral vein adenosine at the same dose had no effect. These data not only support the original work into the neural pathway but, crucially, link this mechanism to the release of the humoral blood-borne mediator. Indeed, several subsequent publications have proven that activation of C-fibre sensory afferents must occur prior to release of the humoral factor.

The downstream effector of RIC following sensory afferent firing, which leads to release of the humoral factor, is not clear. Several studies have implicated a role for the vagus nerve in remote conditioning, although its relationship to release of the humoral factor is not known. The aim of this chapter, therefore, was to investigate further the link between the neural and humoral pathways of RIC, with particular focus on the vagus nerve and intrinsic cardiac ganglia.

## **5.2 Research aims and objectives**

There were two main objectives in this chapter:

1. To investigate the role of the vagus nerve in the release of the humoral RIC mediator;
2. To investigate the role of intrinsic cardiac nerves in the response to the humoral RIC mediator in the myocardium.

### **5.3 Aim 1: The vagus nerve and release of the humoral RIC mediator**

#### **5.3.1 Background**

The vagus nerve is the tenth cranial nerve, and is the most widely distributed cranial nerve in the body. The nerve serves as the main conduit for sensory and motor parasympathetic fibres, with their somata found in two nuclei in the brainstem, the nucleus ambiguus and the dorsal vagal motor nucleus. Emerging from the brain via the jugular foramen and descending into the body as the right and left cervical vagus, the nerve then branches throughout the body and innervates almost every organ, including the heart, lungs, gut, liver and kidneys. Indeed, the nerve was named according to its wide distribution; vagus translates to 'wandering' in Latin. The parasympathetic influence on the body is therefore diverse, ranging from regulating the gut barrier, to sensation in the outer ear, and of course controlling cardiac function.

The extent of vagal innervation in the heart is controversial. Efferent parasympathetic nerves are well documented to widely innervate the atria, and exert control on the atrio-ventricular and sino-atrial nodes to modulate cardiac function [67]. Although previously thought that the ventricles have limited parasympathetic innervation, a growing body of literature has demonstrated a clear presence of fibres stained positive for acetylcholinesterase and a high density of muscarinic M<sub>2</sub> receptors in both ventricles [107]. Indeed, stimulation of the vagus nerve decreased left ventricular contractility independent of its effect on heart rate [216, 378] and, crucially, independent of sympathetic nerve activity [66, 100]. In addition, vagus nerve stimulation inhibited both the chronotropic and inotropic effect of increased sympathetic tone. Cardiac vagal innervation appeared in evolution before sympathetic innervation [107, 338], and the heart in most vertebrates operates under fluctuating levels of inhibitory vagal tone.

The strength of cardiac parasympathetic control is well correlated with risk of death following myocardial infarction. Patients who displayed either low heart rate variability or baroreflex sensitivity carried significant risk of cardiac

mortality following infarction [306]. A subsequent study demonstrated that, even in the absence of cardiovascular disease, the ability to recruit the vagus nerve is a strong indicator of overall mortality, [65]. These data, in addition to the powerful negative inotropy induced by vagus nerve stimulation, led to the idea that the nerve could be targeted as a potential therapeutic agent. Indeed, a growing body of pre-clinical literature has demonstrated that stimulation of the vagus nerve can provide powerful cardioprotection from ischaemia-reperfusion injury [174, 219, 321, 347].

Interestingly, the vagus nerve has recently emerged as one of the key mediators of remote ischaemic conditioning [79, 243]. Section of the vagus nerve at the cervical level consistently blocks the cardioprotection of RIC. The initial conclusion here was that direct cardiac vagal tone was important in the communication of the RIC protective message. However, a recent series of studies demonstrated section of the posterior gastric branch of the vagus nerve also abrogated RIC [25, 242]. The suggestion here is that non-cardiac vagal innervation is important; a cardioprotective factor is released from the gut following increased efferent vagal tone to this region. However, these data presented do not absolutely prove this link, as release of a humoral factor is not measured. This chapter investigates the hypothesis that the humoral mediator is released downstream of vagus nerve activation.

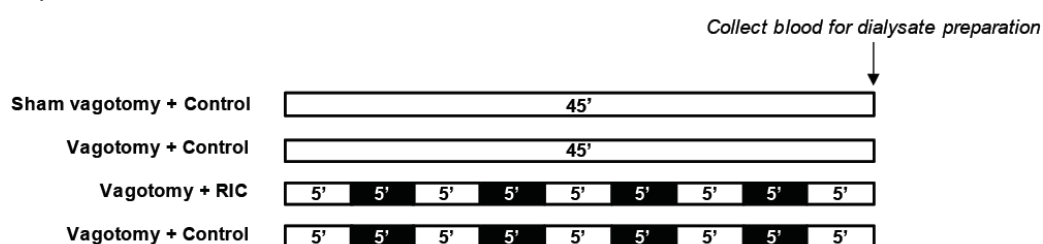
## 5.3.2 Methods

### 5.3.2.1 Bilateral cervical vagotomy

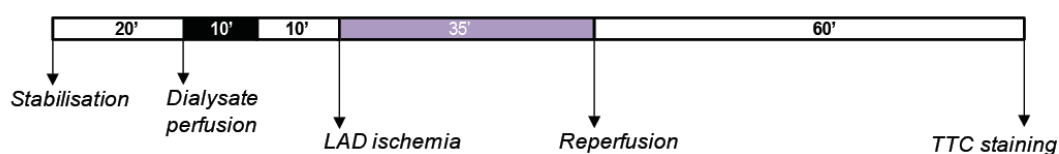
The dialysate model was used for these experiments, for which detailed methods can be found in section 2.3.

Male Sprague-Dawley rats (250-300g) were anaesthetised with 60mg/kg sodium pentobarbital (Animalcare, York, UK) and prepared as described in section 2.3.1. Once stabilised under anaesthesia, the rats were randomised to receive one of four interventions: (1) *Sham vagotomy + Control*, the left and right vagus nerves were isolated at the mid-cervical level, but not severed, and the rat received a sham-RIC protocol; (2) *Sham vagotomy + RIC*, the same as group 1; however, the rat was subjected to RIC; (3) *Vagotomy + Control*, rats were subjected to bilateral cervical vagotomy and received a sham-RIC protocol; and (4) *Vagotomy + RIC*, rats were subjected to bilateral cervical vagotomy and received RIC (Fig. 5-1).

#### i. In vivo procedure



#### ii. Rat Langendorff – recipient dialysate



**Figure 5-1. Experimental protocols used to investigate role of the vagus nerve in release of the humoral RIC mediator.**



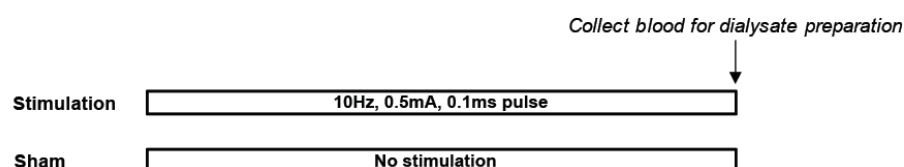
### 5.3.2.2 Stimulation of the posterior gastric branch of the vagus nerve

This experiment was carried out in collaboration with Prof Alex Gourine's laboratory. Svetlana Mastitskaya carried out the in vivo gastric vagus nerve stimulation.

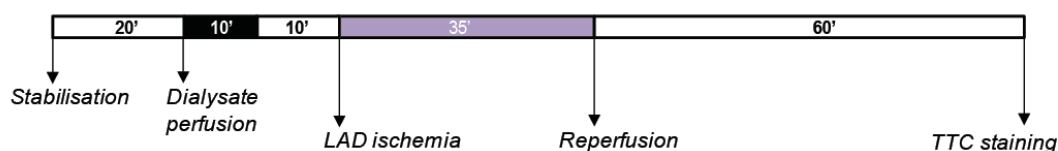
Detailed methods for stimulation of the gastric vagus nerve can be found in a recent publication from Mastitskaya et al. [242].

Briefly, male Sprague-Dawley rats (280-320g) were anaesthetised with sodium pentobarbital (60mg/kg; 10-15 mg.kg<sup>-1</sup>.hr<sup>-1</sup> i.v. maintenance). The posterior gastric branch of the vagus nerve was identified and cleaned of connective tissue. A bipolar silver stimulating electrode was placed on the nerve, with the cathode positioned proximally to the target organ (stomach/gut). The electrode was attached to a constant current stimulator (Model DS3, Digitimer). The stimulator was embedded polyvinylsiloxane dental impression material (Super-Dent, Carlisle Laboratories). The animal was then randomised to receive either electrical stimulation (10Hz, 0.5mA, 0.1ms pulse, 1hr) or a corresponding sham period.

#### i. In vivo procedure - stimulation of posterior gastric branch of the vagus nerve



#### ii. Rat Langendorff – recipient dialysate



**Figure 5-2. Experimental protocols used for posterior gastric vagus nerve stimulation in rats.**

#### **5.3.2.3 Preparation of dialysate**

At the end of each experimental protocol, a clamshell thoracotomy was performed and 9ml blood sampled via right ventricular puncture. The blood was centrifuged in two stages, described in section 2.3.4, to obtain 4ml platelet-free plasma. This was subsequently dialysed across a 12-14kDa membrane (Spectra-Por, Spectrum Laboratories Inc., CA, USA) for 24hr in 200ml of modified Krebs-Henseleit buffer.

#### **5.3.2.4 Perfusion through naïve isolated heart**

Isolated Langendorff-perfused hearts were prepared from male Sprague-Dawley rats (250-300g), as previously described in section 2.2. Following a 20min stabilisation period, the hearts were perfused with the prepared dialysate for 10min, with a 10min washout period. The hearts were then exposed to a 35min left anterior descending coronary artery ischaemia and 60min reperfusion. Infarct size was estimated at the end of the protocol using the Evans blue and triphenyl-tetrazolium chloride method as described in section 2.2.5.

#### **5.3.2.5 Statistical analysis**

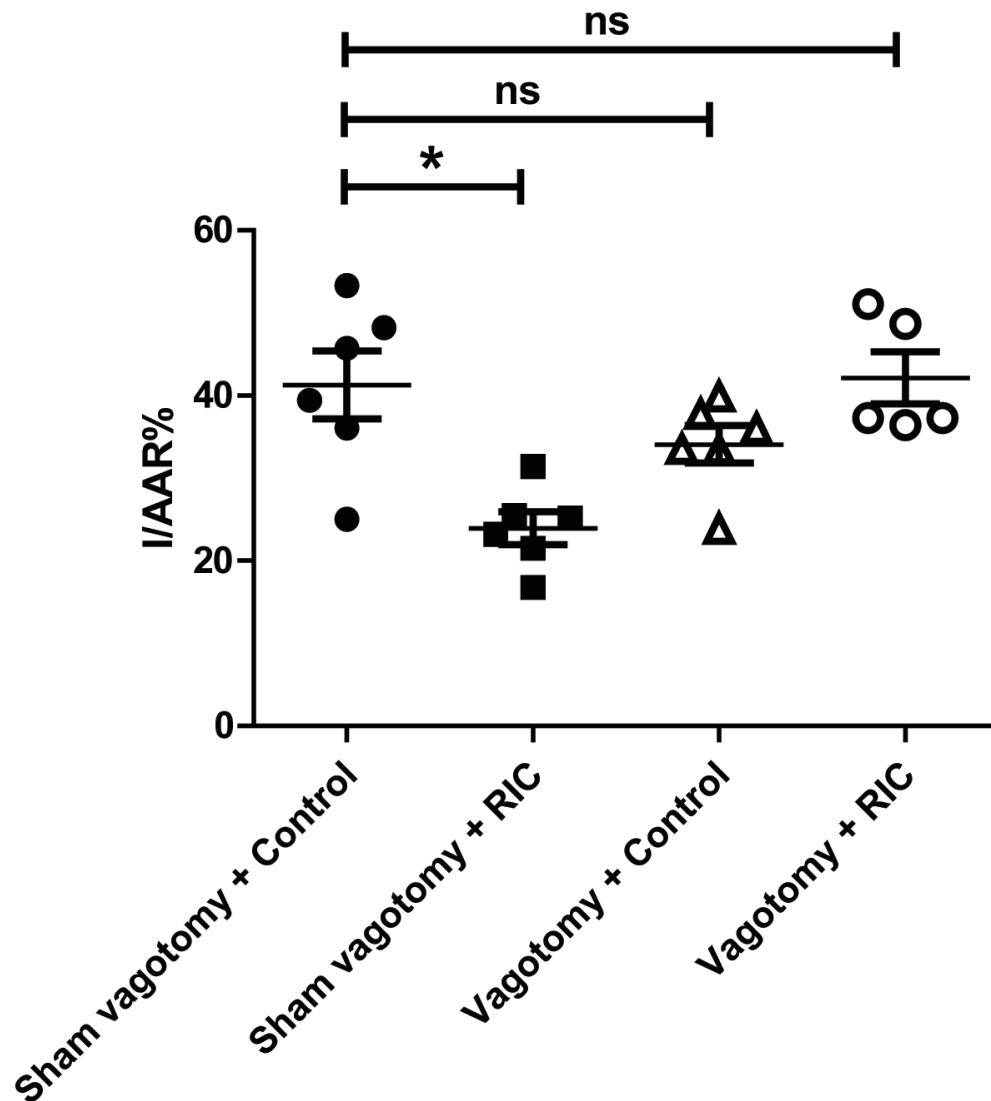
Infarct size was assessed using ImageJ software (version 1.45, National Institutes of Health, USA), and expressed as a percentage of the area-at risk (IS/AAR %). All data are presented as mean  $\pm$  standard error. For the vagotomy study (section 5.3.2.1), the experimental groups were analysed using a one-way analysis of variance (ANOVA). For the second study (5.3.2.2), the two groups were analysed using an un-paired students t-test.

### 5.3.3 Results

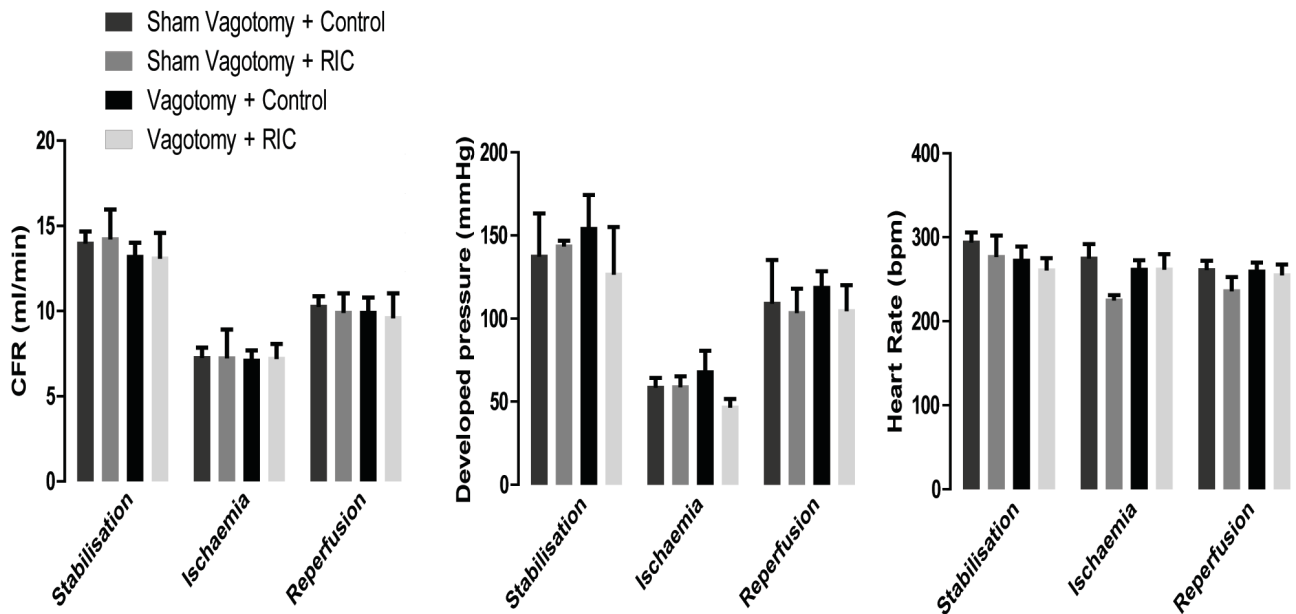
#### **5.3.3.1 *Bilateral cervical vagotomy abrogates release of the humoral RIC mediator***

Sham surgical vagotomy did not influence the efficacy of RIC dialysate to protect a naïve, isolated rat heart (control dialysate from sham-surgery rat: IS/AAR=40.7±6.3% vs RIC dialysate from sham-surgery rat IS/AAR=23.7±3.1,  $p<0.05$ ) (Fig. 3). When the vagus nerve was sectioned bilaterally at the cervical level, RIC dialysate no longer protected the naïve-isolated heart (control dialysate from vagotomised rat: IS/AAR=31.4±2.4% vs RIC dialysate from vagotomised rat: IS/AAR=42.2±3.2%,  $p<0.05$  vs sham-surgery RIC dialysate) (Fig. 5-3).

The hemodynamic data for the naïve-isolated hearts that received dialysate are shown in figure 5-4. Measurements were taken at the beginning of stabilisation, 5min into the index ischaemia and at the end of 60min reperfusion. Those hearts treated with RIC dialysate did not demonstrate improved functional recovery as measured by coronary flow rate (CFR), left ventricular developed pressure (LVEDP) and heart rate (HR). In addition, dialysate prepared following bilateral cervical vagotomy did not affect the functional recovery when perfused through a naïve-isolated heart.



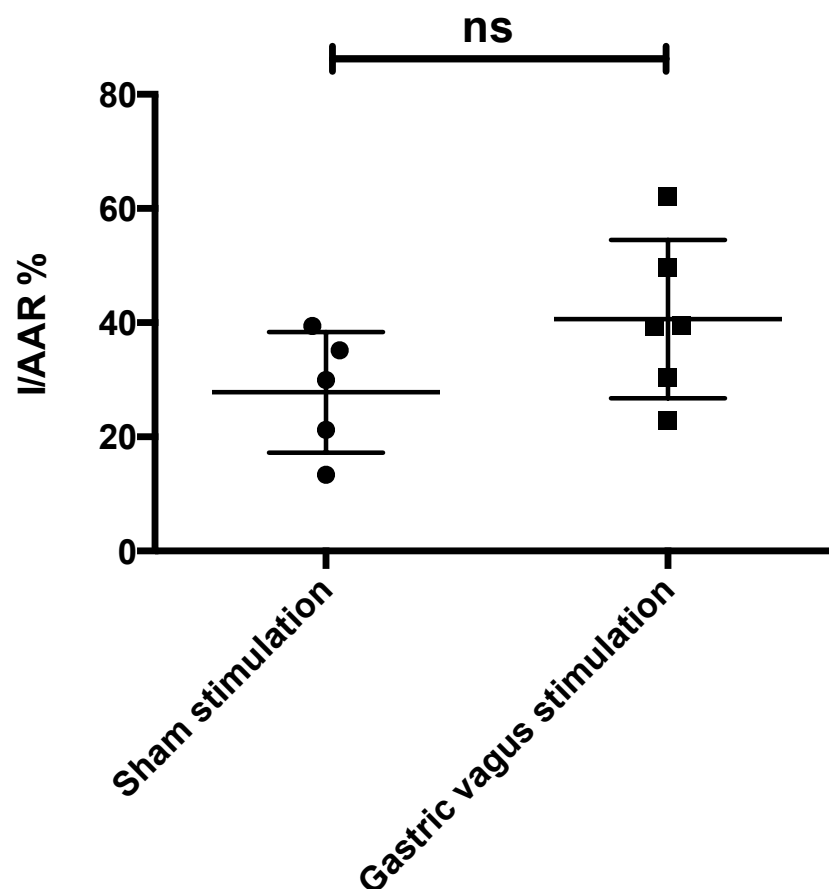
**Figure 5-3: Bilateral cervical vagotomy abrogates dialysate-mediated protection of naïve-isolated rat hearts:** Chart displays left ventricular infarct size as a proportion of the area-at-risk. Dialysate was prepared following *in vivo* vagotomy or sham surgery matched with either RIC or sham protocols. This was perfused through a naïve-isolated heart prior to IRI. Sham vagotomy did not influence the ability of RIC dialysate to induce cardioprotection in the naïve heart. Bilateral cervical vagotomy, however, abrogated RIC-dialysate protection in the naïve heart, suggesting release of the blood-borne humoral mediator was inhibited. Data were analysed via one-way ANOVA with Tukey's post-hoc test, and presented as mean±SEM, with 6-8 animals per group.



**Figure 5-4. Haemodynamic data for bilateral cervical vagotomy experiment:** Measurements were taken following 10min of stabilisation, 5min into the index ischaemia and at the end of reperfusion. (A) coronary flow rate is expressed as ml/min, no significant difference observed between groups. (B) left ventricular developed pressure measurement were obtained via insertion of a fluid-filled balloon into the left ventricle. RIC dialysate did not affect functional recovery of naïve-isolated hearts. (C) heart rate is expressed as beats-per-min, again there were no significant differences between the groups. Data expressed as mean $\pm$ SEM,  $n=6-8$  per group.

### 5.3.3.2 Stimulation of the posterior gastric branch of the vagus nerve

Dialysate prepared following gastric vagus nerve stimulation did not induce a significantly lower infarct size in naïve hearts relative to sham dialysate (Stimulation dialysate I/AAR =  $41 \pm 6$  % vs sham dialysate I/AAR =  $28 \pm 5$  %,  $p = 0.13$ ) (Fig. 5-5). In fact, the dialysate prepared following stimulation appeared more injurious to the naïve heart, although the sham dialysate induced a lower infarct than is normally observed for this ischaemia-reperfusion protocol (see Fig. 3-14).



**Figure 5-5. Dialysate prepared following in vivo stimulation of the posterior gastric branch of the vagus nerve does not protect the naïve rat heart.**

*Dialysate prepared following either posterior gastric vagus nerve stimulation or sham was perfused through naïve rat hearts prior to induction of ischaemia-reperfusion injury. No difference was observed between the two groups (Stimulation dialysate I/AAR =  $41 \pm 6$  % vs sham dialysate I/AAR =  $28 \pm 5$  %,  $p = 0.13$ ). Data presented as mean  $\pm$  SEM and analysed using an unpaired student's *t*-test.*

#### **5.3.4 Discussion**

Our study elucidates a novel aspect to the mechanism of RIC communication. Namely, release of the blood-borne humoral mediator is dependent on prior activation of the vagus nerve. Whilst the importance of the vagus nerve in RIC has been previously reported [79, 80, 242, 243], this study is the first to prove its involvement in the release of the humoral mediator.

Vagus nerve stimulation has been reported in the literature to offer significant cardioprotection from ischaemia-reperfusion injury [79, 243, 322, 347]. Indeed, an important study by Mastitskaya et al demonstrated that activation of a particular group of pre-ganglionic parasympathetic neurones, in the dorsal vagal motor nucleus (DVMN), was sufficient to induce powerful cardioprotection in vivo [243]. In a subsequent and very elegant experiment, the DVMN was genetically silenced using the allatostatin method [243, 363]. This process abrogated RIC-mediated cardioprotection, indicating that this group of preganglionic parasympathetic neurones are fundamental for the communication of the protective message from the conditioned limb to the myocardium. Moreover, a recent study from the same group indicates vagal innervation to the stomach and gut is responsible for RIC communication, suggesting the release of a blood-borne mediator following vagal recruitment [242]. The literature, however, is not fully in agreement with these data [80]. Donato and colleagues demonstrated that cervical but not sub-diaphragmatic vagotomy abrogated RIC, suggesting direct cardiac vagal innervation to be key for RIC communication. The absence of a sham sub-diaphragmatic vagotomy group, however, calls the result into question given the huge abdominal trauma associated with the abdominal surgery. In addition, release of a humoral cardioprotective mediator downstream of vagus nerve activation has not been demonstrated in the literature. The key result from our study, therefore, is that release of the humoral RIC mediator is dependent on prior activation of the vagus nerve. We further conclude that the humoral factor is released from a region of the body innervated by the

vagus nerve below the cervical level. Whether this factor is released following non-cardiac vagal innervation, however, is not clear.

Dialysate prepared following stimulation of the posterior gastric branch of the vagus nerve did not protect a naïve-isolated heart from ischaemia-reperfusion injury. This result is in contrast with those data presented by Mastitskaya and Basalay [25, 242]. Stimulation of the posterior gastric vagal branch induced cardioprotection *in vivo*, and RIC was blocked with the glucagon-like peptide 1 (GLP-1) receptor antagonist exendin(9-39). The main conclusions from these two studies were that RIC requires intact parasympathetic innervation of visceral organs and GLP-1 receptor-mediated signalling. However, there is a lack of clear data indicating a factor is released from the gut following RIC. Data presented in Fig. 5-5 suggests that this parasympathetic branch might not be important in release of the humoral RIC mediator.

Release of the humoral mediator following RIC is dependent on an intact sensory innervation to the conditioned limb [164, 331]. In addition, two important studies demonstrated that direct femoral nerve stimulation, topical application of capsaicin or indeed transcutaneous electrical nerve stimulation generated a plasma dialysate that was able to protect a naïve heart from ischaemia-reperfusion injury [248, 290]. These data suggest the sensory afferent nerve is the sole means of communication from the conditioned limb, and that the factor is released downstream of nerve stimulation. Our study perhaps adds to the model by suggesting that the vagus nerve is the link between sensory nerve activation in the limb and release of the humoral mediator (Fig. 5).

One interpretation of these data is that vagus nerve stimulation may be sufficient to evoke all of the protection conferred by RIC. In the literature, chronic vagal nerve stimulation failed to ameliorate cardiac remodelling or functional capacity in heart failure patients [399]. However, whether vagus nerve stimulation can protect the heart from acute myocardial infarction (AMI) in the clinic is unknown. Perhaps the cuff inflation used currently to induce



RIC could be replaced with non-invasive stimulation of the vagus nerve [330]. A recent clinical study demonstrated that the anesthetic propofol impedes the ability of RIC to protect the heart [198]. Propofol is known to be inhibitory to vagus nerve activity [361], thus suggesting the anesthetic prevents the communication of RIC at the level of the parasympathetic centres in the brainstem. Perhaps RIC should be given while the patient remains conscious, prior to administration of the anesthetic. Secondly, vagal tone is thought to depreciate with age [179]. Therefore, given the high average age of patients who suffer AMI, their diminished vagal tone may reduce the efficacy of RIC. Further study is required to elucidate the effect of age and anesthetics on RIC. In addition, investigation into which branch of the vagus nerve is responsible for inducing release of the factor will help reveal the site of its release and improve the chance of discovering its identity.

## **5.4 Aim 2: The role of intrinsic cardiac ganglia in RIC**

### **5.4.1 Background**

The intrinsic cardiac nervous system was overviewed in detail in section 1.5. This study aimed to extend the neural paradigm of RIC by investigating whether ganglia within the myocardium play a role in the mechanism. There exist within the heart intrinsic neural loops that are able to process sensory information from the myocardial milieu and modulate efferent autonomic output from intrinsic cardiac ganglia, without any necessary input from the central nervous system [16–18, 26]. These neural loops are at risk of destruction following myocardial infarction, and indeed remodel such that the intrinsic cardiac nervous system (ICNS) no longer can function as normal [2, 119, 157, 286]. This is thought to be responsible for the increased risk of arrhythmia following infarction [14]. Whether the ICNS contributes to the myocyte death that occurs during ischaemia-reperfusion, however, is not known.

Initial *in vivo* experiments by Gho et al. demonstrated that ganglionic antagonism, using hexamethonium, blocked the cardioprotection of RIC [101]. Although this suggested a neural pathway, it did not give resolution as to which ganglia were important in the mechanism. In the section above, we gave clear evidence that release of the humoral RIC mediator is dependent on an intact cervical vagal innervation [278]. These data, in combination with two other published studies [25, 242], suggest that non-cardiac vagal innervation is important for RIC, with subsequent release of a humoral cardioprotective factor. The dialysate model (section 3.5) can be used to investigate the transduction of protection in the heart in response to a humoral RIC mediator. An interesting question, therefore, is whether intrinsic cardiac ganglia play a role in response to the humoral mediator. Indeed, given the existence of intrinsic neural reflex loops within the myocardium [15], the Langendorff model may not be the denervated preparation as previously believed. This study investigates the hypothesis that intrinsic cardiac ganglia are recruited by the humoral mediator of RIC as part of the transduction of protection within the myocardium.

## 5.4.2 Methods

### 5.4.2.1 Materials

Hexamethonium (Sigma-Aldrich, Missouri, USA) was employed as a neuronal nicotinic acetylcholine receptor (nAChR) antagonist. Given it has affinity to the muscarinic M2 receptor above 100 $\mu$ M [88], a dose of 50 $\mu$ M was used for this study to aid specificity at nAChRs within cardiac ganglia. Atropine, a muscarinic acetylcholine receptor (mAChR) antagonist, was used at a dose of 100nM. Although often used at micromolar doses in the literature, atropine has a high affinity for mAChRs ( $K_d$ =0.36nM[343]). Thus 100nM was chosen, which has recently been demonstrated to effectively antagonise muscarinic agonism in isolated hearts [170].

### 5.4.2.2 *In vivo procedure and dialysate preparation*

A more detailed description of the dialysate method can be found in section 2.3.

Male Sprague-Dawley rats (250-300g) were used throughout the study. Following anaesthesia with sodium pentobarbitone (60 mg/kg, i.p.), the rats had a small cuff placed around the left hindlimb and were randomised to receive either a sham procedure or remote ischaemic conditioning. RIC was induced as 4 cycles of 5min hindlimb occlusion (200 mmHg cuff inflation) and 5min reperfusion. For the sham procedure, the cuff was not inflated.

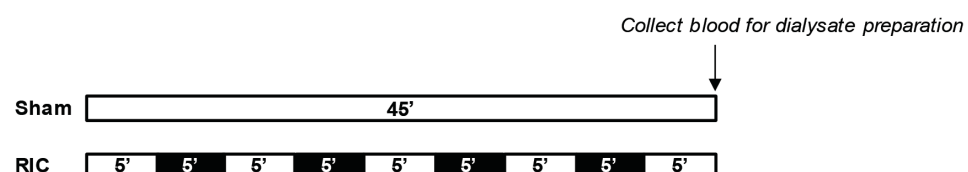
At the end of the protocol, a clamshell thoracotomy was performed and 9ml blood sampled via right ventricular puncture. The blood was centrifuged in two stages, described in section 2.3.4, to obtain 4ml platelet-free plasma. This was subsequently dialysed across a 12-14kDa membrane (Spectra-Por, Spectrum Laboratories Inc., CA, USA) for 24hr in 200ml of modified Krebs-Henseleit buffer.

### 5.4.2.3 *Perfusion of dialysate through a naïve-isolated heart*

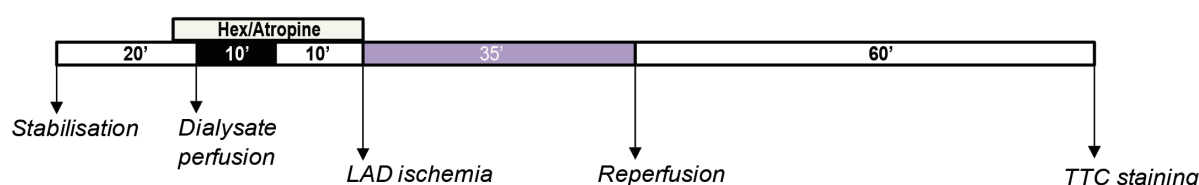
Isolated perfused rat hearts were prepared as described in section 2.2. Hearts were randomly assigned to one of the following 6 groups: (1) *Control dialysate*, hearts received dialysate prepared from an in vivo rat following sham-RIC; (2) *RIC dialysate*, hearts received dialysate prepared from an in

vivo rat following in vivo RIC; (3) *Control dialysate + Hexamethonium (50 $\mu$ M)*; (4) *RIC dialysate + Hexamethonium*; (5) *Control dialysate + Atropine (100nM)*; (6) *RIC dialysate + Atropine (100nM)*. In groups 3-6, drug perfusion was initiated 5-min prior to and for the duration of dialysate treatment. All hearts were perfused with the dialysate for 10-min, with a subsequent 10-min washout period, prior to a 35-min LAD index ischaemia and 60-min reperfusion.

i. *In vivo procedure*



ii. *Rat Langendorff – recipient dialysate pharmacology*



**Figure 5-6. Experimental protocol.** (i) Male Sprague-Dawley rats were randomised to receive either RIC (4x5min hindlimb ischaemia-reperfusion) or sham. Dialysate was prepared following each intervention. (ii) Isolated hearts were treated with either hexamethonium (50 $\mu$ M) or atropine (100nM) for 5min prior to and the duration of dialysate perfusion/washout. All hearts received 35 min left anterior descending coronary artery ischaemia and 60 min reperfusion. Following each Langendorff experiment, infarct size (IS) was determined using TTC staining.

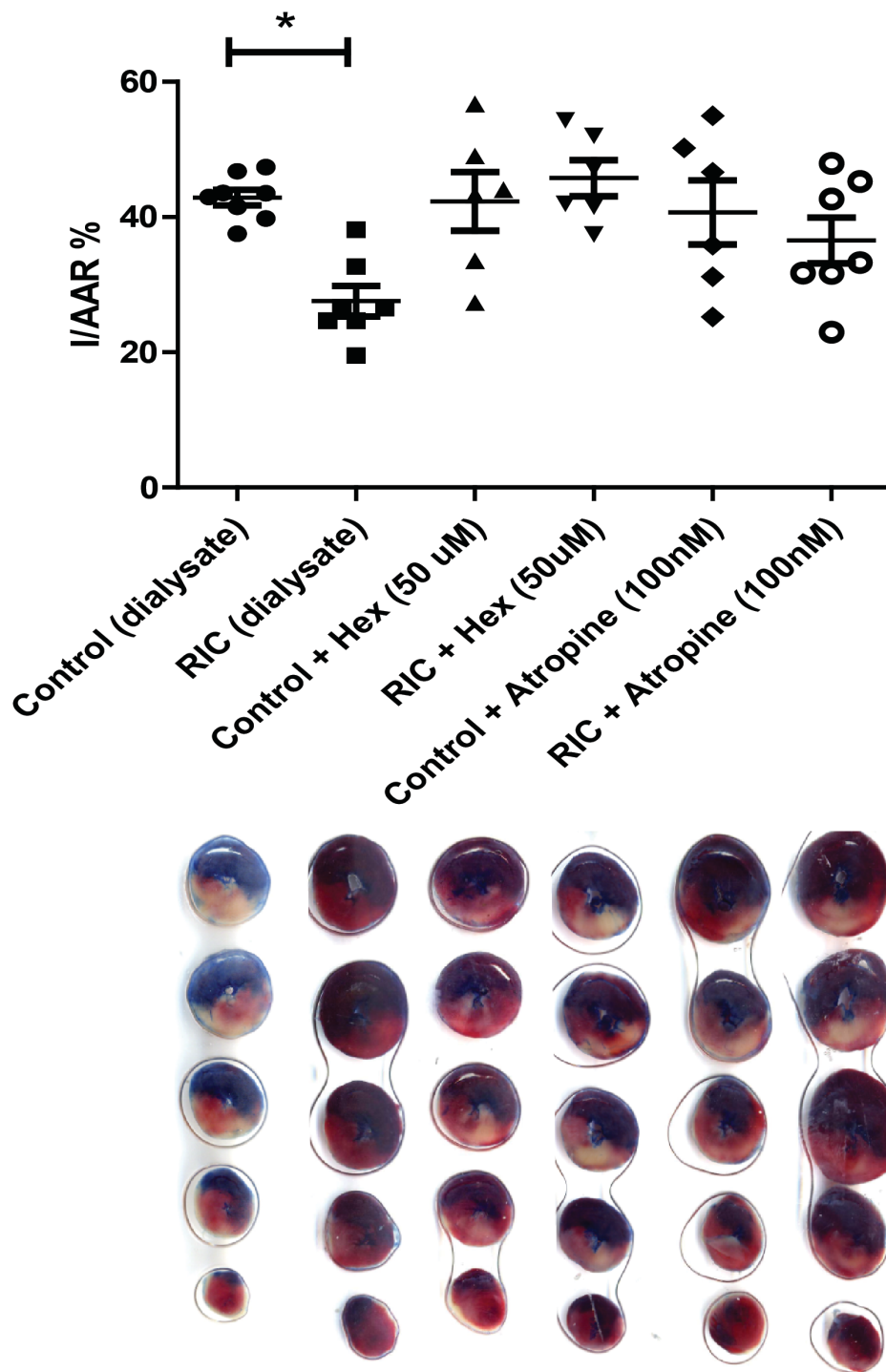
#### 5.4.2.4 Statistical analysis

Infarct size was assessed using ImageJ software (version 1.45, National Institutes of Health, USA), and expressed as a percentage of the area-at risk (IS/AAR %). All data are presented as mean  $\pm$  standard error. The experimental groups were analysed using a one-way analysis of variance (ANOVA),  $p < 0.05$  was considered significant.

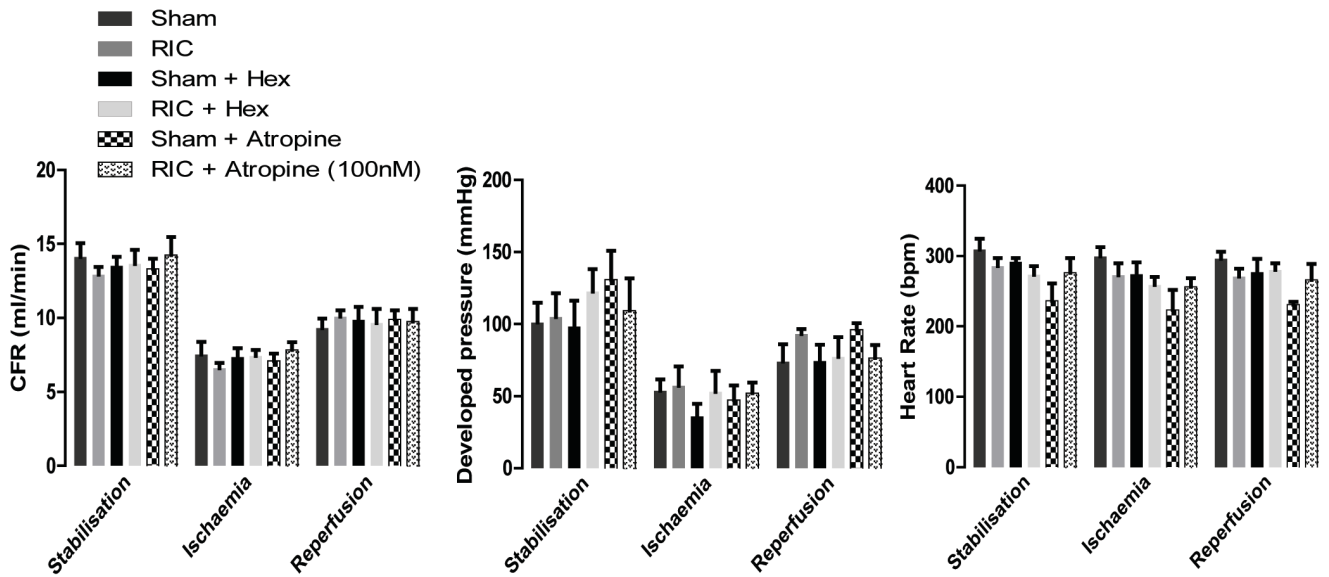
### 5.4.3 Results

Figure 4 displays the infarct size chart from naïve-isolated rat hearts subjected to dialysate in the presence or absence of either the ganglionic blocker hexamethonium, or the muscarinic antagonist atropine. Those hearts treated with RIC dialysate in the absence of either drug induced powerful cardioprotection (Sham dialysate IS/AAR=40.1±1.2 vs RIC dialysate IS/AAR=27.6±2.3,  $p<0.05$ ). In the presence of hexamethonium (50µM), RIC dialysate was no longer able to protect naïve hearts (Sham dialysate + Hex IS/AAR=42.3±4.3 vs RIC dialysate + Hex IS/AAR=45.8±2.7%,  $p>0.05$  vs sham dialysate). The muscarinic antagonist atropine (100nM) also abrogated RIC dialysate-mediated cardioprotection (Sham dialysate + Atropine IS/AAR=40.7±4.8% vs RIC dialysate + Atropine IS/AAR=36.5±3.4%,  $p>0.05$  vs sham dialysate) (Fig. 5-6).

The hemodynamic data again indicated that RIC dialysate did not significantly influence functional recovery of naïve hearts relative to control. In addition, neither hexamethonium nor atropine affected functional recovery of the isolated hearts (Fig. 5-7).



**Figure 5-7. Hexamethonium and atropine abrogate dialysate-mediated cardioprotection:** Isolated rat hearts were perfused with dialysate prepared following *in vivo* RIC or sham procedures. RIC dialysate significantly protected the naïve heart from IRI relative to sham. When the naïve heart was pretreated with either the ganglionic antagonist hexamethonium (50 $\mu$ M) or the muscarinic antagonist atropine (100nM) abrogated this protection. Data expressed as mean $\pm$ SEM, n=6-8 per group.



**Figure 5-8 Haemodynamic data for the intrinsic cardiac ganglia experiment:** Measurements were taken following 10min of stabilisation, 5min into the index ischaemia and at the end of reperfusion. (A) coronary flow rate is expressed as ml/min, no significant difference observed between groups. (B) left ventricular developed pressure measurement were obtained via insertion of a fluid-filled balloon into the left ventricle. RIC dialysate did not affect functional recovery of naïve-isolated hearts. (C) heart rate is expressed as beats-per-min, again there were no significant differences between the groups. Data expressed as mean±SEM, n=6-8 per group.

#### 5.4.4 Discussion

These data provide the first evidence that intrinsic cardiac neural pathways are important in the mechanism of remote ischaemic conditioning. Growing evidence suggests that cardiac neural control is hierarchical. Indeed, sensory information from the heart can be received by: (1) central nervous control from medullary autonomic centres in the brain, which provide information to the heart via autonomic efferent pre-ganglionic neurons; (2) intrathoracic extracardiac ganglia; (3) intrinsic cardiac ganglia [12, 15–18, 152, 180, 230]. Intrinsic cardiac ganglia are able to process sensory information from the myocardium and directly activate efferent postganglionic nerve firing from intrinsic cardiac ganglia, thus neural control of the heart can occur without any extracardiac input [26, 180, 181]. The Langendorff perfused isolated heart is traditionally thought of as a denervated preparation, however in light of recent studies described above, as well as the results from this paper, it appears that intrinsic neural loops remain intact in the isolated heart and continue to play an important role in its function and ability to withstand ischaemia-reperfusion injury.

Transmission of a sensory message in a pre-ganglionic synapse within intrinsic cardiac ganglia is governed via the release of acetylcholine into the synaptic cleft, which will bind to and activate nicotinic acetylcholine receptors (nAChR), on the post-ganglionic nerve, causing a depolarisation and initiation of the nerve impulse [34, 71, 106]. Hexamethonium will antagonise the nAChR, thus preventing transmission of information at the ganglia. The observation, therefore, that hexamethonium abrogates dialysate-mediated protection suggests the humoral factor recruits intrinsic cardiac ganglia as an essential process in the induction of cardioprotection. Muscarinic acetylcholine receptors (mAChR) are present primarily on the sarcolemma of cardiomyocytes [44]. They respond to acetylcholine released from parasympathetic post-ganglionic neurons, which innervate the ventricles [67, 178, 307, 348]. Thus, the observation that atropine, a mAChR antagonist, abrogates dialysate-mediated cardioprotection suggests the humoral factor induces increased intrinsic post-ganglionic parasympathetic nerve outflow.



Previous evidence has demonstrated that hexamethonium and atropine abrogate in vivo RIC in rats [79, 101, 243], although the literature is not in full agreement [366]. In addition, evidence that the endothelium can be protected by RIC discounts the importance of intrinsic nerves in responding to the humoral mediator [186]. However, the protection offered to endothelium by RIC may be obtained via a different mechanism relative to the myocardium. Our data suggest the intrinsic cardiac ganglia play an important role in this setting.

#### **5.4.5 Limitations and future directions**

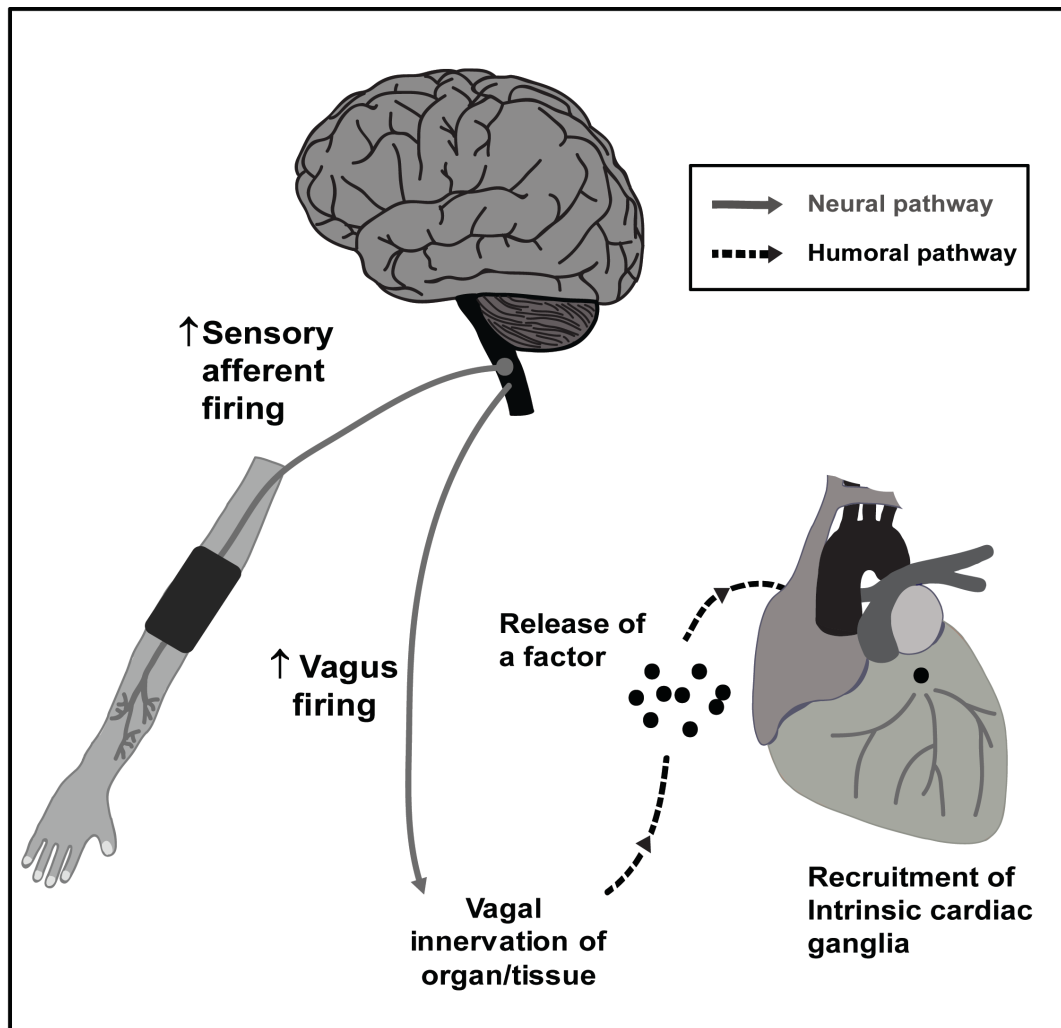
These data are limited by both the specificity of each drug to the desired receptor, and the localisation of each receptor within the heart. Hexamethonium is known to have specificity for muscarinic as well as nicotinic acetylcholine receptors. Although the dose used was chosen to ensure minimal affinity to muscarinic receptors, one cannot be sure of full selectivity for nicotinic receptors. Moreover, nicotinic receptors are not only expressed in neuronal ganglia within the heart, but also in cardiomyocytes [87]. Thus, it is possible that hexamethonium is exerting its inhibition of cardioprotection through direct action on cardiomyocytes rather than intrinsic ganglia.

Future work could include use of genetic techniques to further dissect the mechanism of RIC. For example, development of murine models of  $G_{i/o}$  protein knockout in both cardiomyocytes and intrinsic ganglia. One could then investigate whether cardioprotection by RIC was still effective in each model, the hypothesis being that  $G_{i/o}$  knockout within intrinsic ganglia would abrogate RIC.

## 5.5 Summary

Data presented in this chapter, in combination two other recent studies, add important information to the RIC mechanism. First, we extended the neuro-humoral hypothesis by demonstrating release of the humoral RIC mediator is dependent on prior vagus nerve activation. Second, the humoral RIC mediator exerts protection in the myocardium via activation of intrinsic cardiac ganglia. Our data are supported by two recent studies from Prof Alex Gourine [25, 242], where non-cardiac vagal innervation and subsequent release of glucagon-like peptide 1 (GLP-1) was necessary for RIC-mediated cardioprotection. Moreover, the GLP-1 analogue, exenatide, was able to induce powerful cardioprotection by an atropine-sensitive mechanism. This suggests activation of intrinsic cardiac ganglia by GLP-1 following RIC, which supports our data.

These findings may appear initially as an inefficient mechanism of communication; why, for example, does the vagus nerve not communicate directly to the heart? However, it is important to remember that RIC can protect several other organs in addition to the heart. In addition, the evidence that a denervated/transplanted heart can be protected by RIC argues against a role for direct vagal innervation [196]. We therefore suggest the following paradigm for RIC communication; brief cycles of ischaemia in the limb induce C-fibre sensory afferent firing, leading to increased vagal tone to a non-cardiac organ, triggering release of a humoral blood-borne factor that subsequently induces cardioprotection via activation of intrinsic ganglia (see Fig. 5-9 for schematic).



**Figure 5-9. Schematic of proposed mechanism of RIC communication (figure adapted from that in [276]):** Serial inflations and deflations of a cuff around the upper limb will activate sensory afferent nerves [331]. These in turn will convey their message to autonomic regions of the brainstem, leading to increased systemic efferent vagal tone. The vagus nerve will innervate an organ remote from the heart, which induces release of a dialysable cardioprotective factor less than 12-14kDa in size. This factor will move to the heart via the blood and induce a protective phenotype within the myocardium, in part via recruitment of intrinsic cardiac ganglia.

## Chapter 6 INTRINSIC CARDIAC GANGLIA AND ACETYLCHOLINE ARE IMPORTANT IN THE MECHANISM OF CLASSICAL ISCHAEMIC PRECONDITIONING

The work presented in this chapter has been recently published. Chapter 10 contains a copy of the published manuscript.

***Pickard JMJ, Burke N, Davidson SM, Yellon DM (2017) Intrinsic cardiac ganglia and acetylcholine are important in the mechanism of ischaemic preconditioning. Basic Res Cardiol 112:11. doi: 10.1007/s00395-017-0601-x***

### 6.1 Introduction

Ischaemic preconditioning (IPC) is a powerful cardioprotective phenomenon, whereby brief cycles of ischaemia to a coronary bed renders it less susceptible to subsequent ischaemia-and-reperfusion-mediated infarction [255]. Indeed, IPC has emerged as a highly conserved cardioprotective intervention, effective in many mammalian species via a similar mechanistic pathway (see meta-analysis [368] and recent review [134]). Although studies have reported protection by classic IPC in the setting of cardiac surgery [160], it is not practical to be used clinically in either this setting or indeed in the in the setting of acute myocardial infarction (AMI). However, the potency of cardioprotection offered by classic IPC provides a very useful tool to better understand the physiological basis of cardioprotection.

There are three interesting mechanistic traits of classic IPC: (i) there are several **triggers** which initiate the protective reflex, (ii) a **threshold** exists that must be surpassed in order for protection to occur and (ii) the presence of an **effector** signalling pathway within the cardiomyocyte is necessary. The triggers for IPC are several small molecules, released in the heart following the brief cycles of ischaemia. These include adenosine [226], opioids [313] and bradykinin [357]. Blocking the receptor for one of these molecules abrogates IPC, however this can be overcome by additional cycles of brief ischaemia [105]. Thus, IPC involves release of multiple trigger molecules that, via receptor activation, converge on a common cardioprotective

signalling pathway. This indicates that there exists a threshold that must be reached to induce cardioprotection, relating to the strength of the IPC stimulus. Protection is observed after a single ischaemic episode of 2.5 min, but not after shorter periods [209]. Moreover, the strength of protection seems to increase with the number of cycles of IPC, such that three cycles of 5 min ischaemia affords greater protection than one [368]. Unsurprisingly, very long IPC ischaemic cycles no longer provide any cardioprotection [368]. Finally, the **effector** pathway, which involves activation of several well characterised pro-survival signalling pathways within the cardiomyocyte [131, 132] and renders the cell resistant to death. Perhaps the most interesting aspect of IPC is that, despite application of the intervention prior to the index ischaemia, the majority of protection is provided against reperfusion injury. Indeed, we have demonstrated that inhibition of the RISK pathway at the point of reperfusion abrogated IPC-mediated cardioprotection [129]. This necessitates a **memory phase**, during which the myocardium “remembers” the protective intervention prior to its employment at reperfusion. Indeed, the initial window of protection offered by IPC lasts up to 2 hours prior to the index ischaemia [206, 220]. The mechanism of this apparent memory phase is as yet unclear.

Neural control of the heart is typically thought to be mediated by regions in the brainstem and spinal cord. Indeed, the autonomic ganglia that reside within the thorax and myocardium have long been thought of as monosynaptic relay stations, which serve to confer the complex processing and efferent output of the central nervous system. In fact, there exists a complex hierarchy of cardiac neural control, with sensory afferent nerves of cardiac origin found not just in central nervous system (CNS) ganglia, but also intrathoracic and intracardiac ganglia [13]. Intrinsic cardiac ganglia are thus able to process sensory information and control efferent post-ganglionic autonomic firing within the heart, in the absence of any central modulation [11]. Moreover, a recent study revealed a heterogeneous population of intrinsic cardiac nerves, in particular local circuit neurons, which respond to a variety of stimuli and can influence cardiac function on a beat-to-beat basis without CNS influence [26]. Thus, complex neural processing occurs within

the heart, not just in response to central efferent input, but also sensory afferent information from the myocardium. Whether these reflexes remain intact in the Langendorff isolated heart preparation, however, is yet to be investigated.

The massive sensory and ischaemic trauma associated with myocardial infarction (MI) induces dynamic morphological and phenotypic remodelling of the intrinsic cardiac nervous system, not limited to the infarcted region [119]. A 'neural sensory border zone' of infarction appears, with those afferents within the infarcted region becoming less sensitive, and those in the border and remote regions preserved or enhanced [286]. The influence of this neural remodelling is not yet clear, although it is thought to contribute to ventricular arrhythmogenesis [57]. In addition, these effects occur over a period of weeks following infarction, thus the acute influence of the intrinsic cardiac nervous system on IRI is yet to be understood.

## **6.2 Research aims and objectives**

The intrinsic cardiac nervous system has recently been implicated in the cardioprotection induced by remote ischaemic conditioning and vagus nerve stimulation [27, 278]. Here, we present two separate studies designed to investigate the importance of intrinsic cardiac ganglia in classical IPC; the first using an isolated perfused heart preparation, the second using a model of IPC via coronary effluent transfer.

### 6.3 Methods

#### *Materials*

Dose justification was given in detail for hexamethonium and atropine in a recent publication from the same authors [278]. Briefly, Hexamethonium (Sigma-Aldrich, Missouri, USA) was employed as a neuronal nicotinic acetylcholine receptor (nAChR) antagonist, at 50  $\mu$ M, to achieve specificity at nAChRs within cardiac ganglia. Atropine, a muscarinic acetylcholine receptor (mAChR) antagonist, was used at a dose of 100 nM based on its affinity to the receptor ( $K_d=0.36$  nM).

#### *Animals*

All animals received humane care in accordance with the United Kingdom (Scientific Procedures) Act of 1986. Male Sprague-Dawley (SD) rats were bred at a central animal unit in University College London and were used at a weight of 250-300 g throughout the study.

#### *Langendorff perfused heart preparation*

Rats were anaesthetised with an upper left quadrant intraperitoneal injection of sodium pentobarbitone (60 mg/kg) (Animalcare, York, UK). Hearts were quickly excised via a clamshell thoracotomy and the aorta cannulated on a Langendorff apparatus to allow for retrograde perfusion of modified Krebs-Henseleit buffer (118 mM NaCl, 25 mM NaHCO<sub>3</sub>, 11 mM D-glucose, 4.7 mM KCl, 1.22 mM MgSO<sub>4</sub>·7H<sub>2</sub>O, 1.21 mM KH<sub>2</sub>PO<sub>4</sub> and 1.84 mM CaCl<sub>2</sub>·2H<sub>2</sub>O). The buffer was warmed to 37.5°C and gassed with 95% O<sub>2</sub> / 5% CO<sub>2</sub> to obtain a pH of 7.35 - 7.45) [for detailed methods see 1]. A fluid-filled latex balloon was inserted into the left ventricle to allow for measurement of functional parameters, including heart rate (HR) and left ventricular developed pressure (LVEDP). Coronary flow rate (CFR) was recorded throughout the protocol and the temperature of the heart was maintained at 37.0  $\pm$  0.5°C. Finally, a 3-0 Mersilk suture (Ethicon, Edinburgh, UK) was inserted through the heart to surround the left anterior descending (LAD)

coronary artery. All hearts received a 35 min LAD ischaemia and 60 min reperfusion.

## **Study 1**

### *Classic ischaemic preconditioning*

Two separate experiments were designed to investigate intrinsic cardiac nerves in classic ischaemic conditioning. The first experiment tested involvement of intrinsic cardiac ganglia in IPC, via use of the nicotinic acetylcholine receptor (nAChR) antagonist, hexamethonium. Isolated perfused rat hearts were randomly assigned to one of the following 6 groups: (1) *Sham IPC*, hearts received a 40-min stabilisation period; (2) *Control + Hexamethonium (50  $\mu$ M)*, hearts received a 10-min stabilisation period followed by 35 min perfusion with 50  $\mu$ M hexamethonium. (3) *IPC1*, hearts received one cycle of 5-min global ischaemia followed by a 5-min reperfusion prior to index ischaemia (4) *IPC3*, hearts received 3 cycles of 5-min- global ischaemia with intermittent 5-min- reperfusion immediately prior to index ischaemia; (5) *IPC1 + Hexamethonium (50 $\mu$ M)*, same as group 3 however the hearts were treated with hexamethonium for 5-minutes prior to and the duration of the 1-cycle preconditioning; (6) *IPC3 + Hexamethonium (50 $\mu$ M)*, same as group 4 however the hearts were treated with hexamethonium for 5-min prior to and the duration of the 3-cycle preconditioning.

The second study looked at the importance of muscarinic acetylcholine receptors (mAChR) in classic IPC, via use of the drug atropine. Hearts were randomly assigned to one of 3 groups: (1) *Control + Atropine (100nM)*, hearts received a 10min stabilisation followed by 35-min perfusion with 100nM atropine; (2) *IPC3*, hearts received 3-cycles of 5-min global ischaemia with intermittent 5-min reperfusion immediately prior to index ischaemia; (3) *IPC3 + Atropine (100nM)*, same as group 2 however hearts were perfused with hexamethonium for 5-min- prior to and the duration of the IPC protocol.



All hearts subsequently received 35-min LAD ischaemia and 60-min reperfusion. At the end of the protocol, hearts were analysed for infarct size using methods described below.

## Study 2

### *Classic IPC with coronary effluent transfer to naïve isolated hearts*

This study uses a model first pioneered by the Przyklenk laboratory [76], and has been used in several subsequent publications by different groups [43, 215]. Although it is described in the literature as more similar to remote ischaemic preconditioning, in fact it likely reflects the humoral aspect to classical preconditioning. That is, it enables one to investigate the factors released by the heart following IPC. RIC is now generally agreed to occur via a more complex neuro-humoral pathway [278].

In the first part of the experiment, coronary effluent was collected from isolated perfused donor rat hearts, randomised into one of the following two groups: (1) *Donor Control* hearts underwent 30-min of perfusion during which effluent was collected; (2) *Donor IPC* hearts received three cycles of 5-min global ischaemia with intermittent 5-min reperfusion, during which effluent was collected.

In the second part, recipient hearts were perfused with effluent (from above) for 10-min, following 30-min of stabilisation, immediately prior to 35-min LAD ischaemia and 60-min reperfusion. These recipient hearts were randomised to one of four groups: (1)  $C_{eff}$ , hearts received 10-min perfusion of donor control effluent immediately prior to ischaemia; (2)  $IPC_{eff}$ , hearts received a matched 10-min perfusion of donor IPC effluent; (3)  $C_{eff} + Hex$ , the same as group 1, however hearts were perfused with hexamethonium (50  $\mu$ M) for 5-min prior to and the duration of effluent perfusion; (4)  $IPC_{eff} + Hex$ , same as group 2 however hearts were perfused with hexamethonium for 5-min prior to and the duration of effluent perfusion. Following reperfusion, all hearts were analysed for infarct size using methods described below.

### *Acetylcholine assay*

A Choline/Acetylcholine Assay Kit (Abcam, UK) was used to measure the concentration of acetylcholine in effluent collected following IPC (3x5min global ischaemia-reperfusion) or corresponding control period, as described above. The assay was carried out in accordance with the instructions provided by the manufacturer. Briefly, via the use of acetylcholinesterase, the level of free and total choline was measured in each sample, enabling an estimation of the concentration of ACh within the sample.

### *Classic IPC with coronary effluent transfer to naive isolated cardiomyocytes*

In order to ascertain the role of the intrinsic cardiac nerves in classic IPC we undertook a series of studies using the isolated cardiomyocyte, where nerves are not present. Isolation of adult male Sprague-Dawley rat (250-300 g) cardiomyocytes was performed using a previously described protocol [136]. Cells were plated on laminin-coated 35 mm dishes (VWR international, PA, USA) and left to stabilise for 24-hr prior to use. Dishes were assigned to one of the following groups: (1) *Normoxia*, cells were left in M119 media for the duration of the protocol; (2) *Vector control*, cells were stimulated for 10 min with Krebs-Henseleit buffer; (3) *C<sub>eff</sub>*, cells were stimulated for 10-min with control effluent; (4) *IPC<sub>eff</sub>*, cells were stimulated for 10-min with IPC effluent; (5) *NECA*, cells were stimulated with the adenosine A2B receptor agonist, 5'-N-ethylcarboxamidoadenosine (NECA). Following stimulation (groups 2-5), cells were treated with hypoxic buffer [NaCl 127.8 mM, 14.8 mM KCl, KH<sub>2</sub>PO<sub>4</sub> 1.2 mM, MgSO<sub>4</sub> 1.2 mM, NaHCO<sub>3</sub> 2.2 mM, CaCl<sub>2</sub> 1 mM, Na. lactate 10 mM, gassed with 5%CO<sub>2</sub>-95% N<sub>2</sub> to achieve pH6.4], and placed into a sealed hypoxic chamber (Billups-rothenberg, CA, USA) filled with 5%CO<sub>2</sub>-95% N<sub>2</sub> gas mix. Hypoxia was continued for 3 hr at 37°C, at which point the cells were removed from the chamber and treated with normoxic buffer [Glucose 10 mM, NaCl 118 mM, KCl 2.6 mM, KH<sub>2</sub>PO<sub>4</sub> 1.2 mM, MgSO<sub>4</sub> 1.2 mM, NaHCO<sub>3</sub> 22 mM, CaCl<sub>2</sub> 1 mM, gassed with 5%CO<sub>2</sub>-95% O<sub>2</sub> to achieve pH7.4] to simulate reperfusion. The reoxygenation was continued for 1hr, at

which point the proportion of cell death was measured via propidium iodide staining and confocal microscopy (previously described here [352]).

#### *Infarct size assessment*

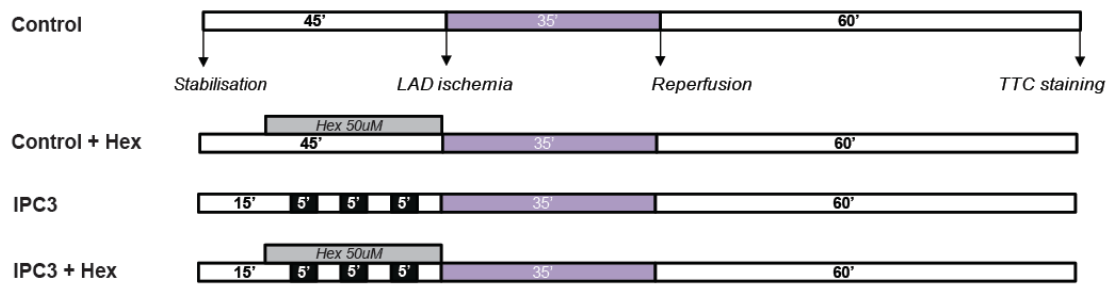
Infarct size of each isolated heart in the above experiments was calculated using the following methods, described in detail previously [30]. Briefly, at the end of the reperfusion period, the LAD suture was re-tightened and 1ml of 0.25% Evans blue dye was perfused through the heart in order to delineate the area-at-risk of infarction. The hearts were then frozen at -20°C before being sectioned into 5-transverse slices and stained for viable tissue by immersion in 1% triphenyl-tetrazolium chloride at 37°C for 15-min. Following fixation in 10% formalin for 24-hr, the sections were digitally scanned to a computer for analysis. Analysis of infarct size (IS) as a proportion of area at risk (AAR) was calculated via planimetry using imageJ software (version 1.45, National Institutes of Health, USA).

#### *Statistical analysis*

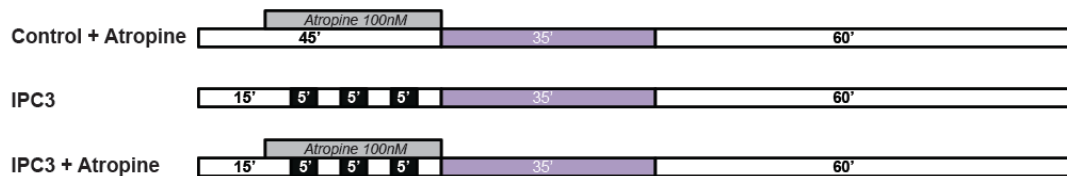
Data groups were first analysed for normality using the Kolmogorov-Smirnov test. Statistical differences between two groups were analysed using a student's t-test and more than two groups using a one-way analysis of variance (ANOVA) with Tukey's multiple comparison post-test. All data is presented as mean  $\pm$  standard error of the mean (SEM). Data groups were classed as significantly different with a p value less than 0.05. Notation of significance is described in figure legends. Analyses were performed using GraphPad Prism version 5 for Windows (CA, USA).

## Study 1

### A. IPC + hexamethonium protocols

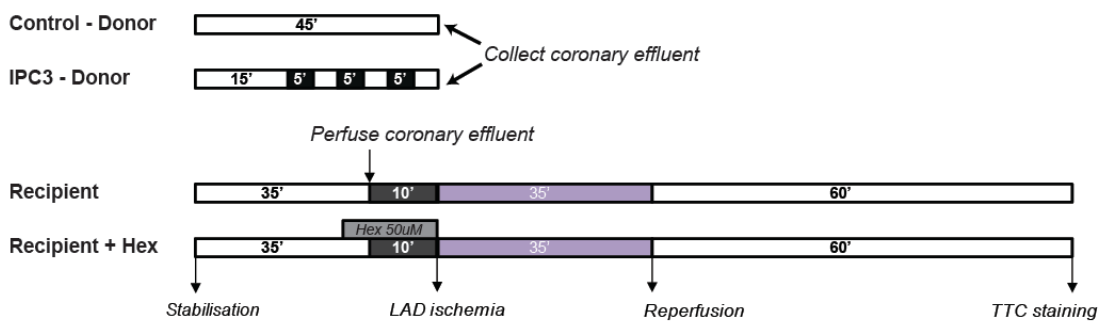


### B. IPC + atropine protocols

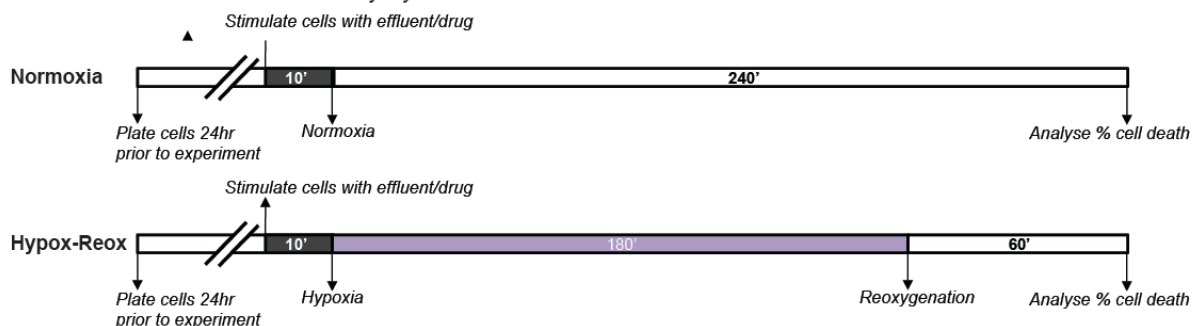


## Study 2

### A. IPC effluent - naïve isolated hearts



### B. IPC effluent - naïve isolated cardiomyocytes



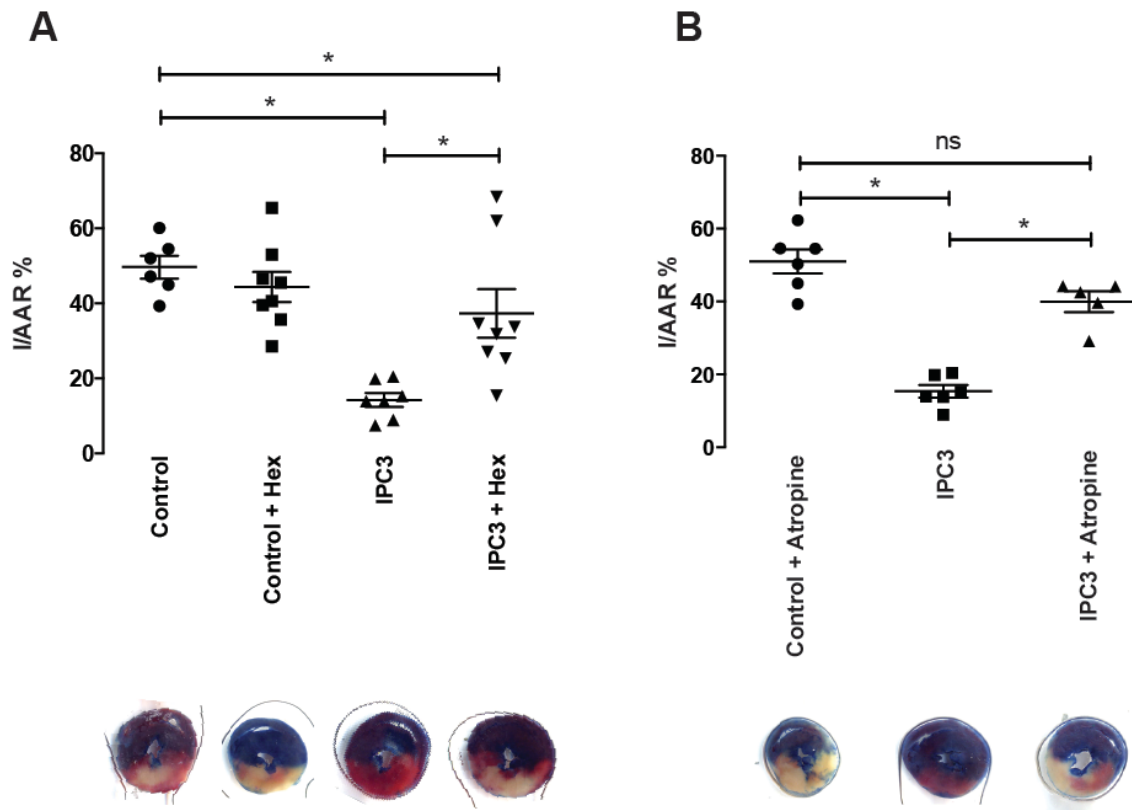
**Figure 6-1** Schema detailing the experimental protocols: Rat hearts were subjected to 35 min LAD ischaemia and 60 min reperfusion. Preconditioning was induced by three cycles of 5 min global ischaemia-reperfusion. Study one, Hexamethonium (1A) and atropine (1B) were perfused through the heart for 5 min prior to and for the duration of the conditioning protocol. Study two, coronary effluent was collected from isolated hearts either following IPC or control, and subsequently perfused through (2A) a naïve isolated heart and prior to index ischaemia. Again, hexamethonium was perfused through the recipient heart for 5 min prior to and the duration of effluent perfusion. Coronary effluent was used to stimulate isolated cardiomyocytes (2B) prior to hypoxia-reoxygenation injury.

## 6.4 Results

### 6.4.1 Study 1: Classic ischaemic preconditioning is abrogated by hexamethonium and atropine

In our isolated perfused rat heart model we demonstrated that three cycles of IPC (IPC3) was effective at reducing infarct size relative to control (IS/AAR =  $14 \pm 2\%$  vs control IS/AAR =  $48 \pm 3\%$ ,  $p < 0.05$ ) (Fig. 2A). The nAChR antagonist, hexamethonium ( $50 \mu\text{M}$ ), almost fully abrogated this cardioprotection (IS/AAR =  $37 \pm 7\%$ ,  $p > 0.05$  vs control).

In the second part of this study, the muscarinic antagonist, atropine, was used to investigate the pathway downstream of intrinsic ganglia.  $100 \text{ nM}$  atropine did not affect infarct size (IS/AAR =  $51 \pm 3\%$ ), however it abrogated the cardioprotection induced via 3-cycles of IPC (IS/AAR =  $40 \pm 3\%$  vs IPC3 =  $15 \pm 2\%$ ) (Fig. 2B).



**Figure 6-2: Hexamethonium and Atropine abrogate ischaemic preconditioning.** (A) Hexamethonium abrogates preconditioning induced by both one and three cycles of IPC ( $n=6-8$  per group,  $* = p<0.05$  vs control); (B) Atropine also abrogates the protection afforded by IPC3 ( $n=6$  per group except IPC+Atropine where  $n=5$ ,  $* = P<0.05$  vs Control+Atropine). Data presented as mean  $\pm$  SEM.

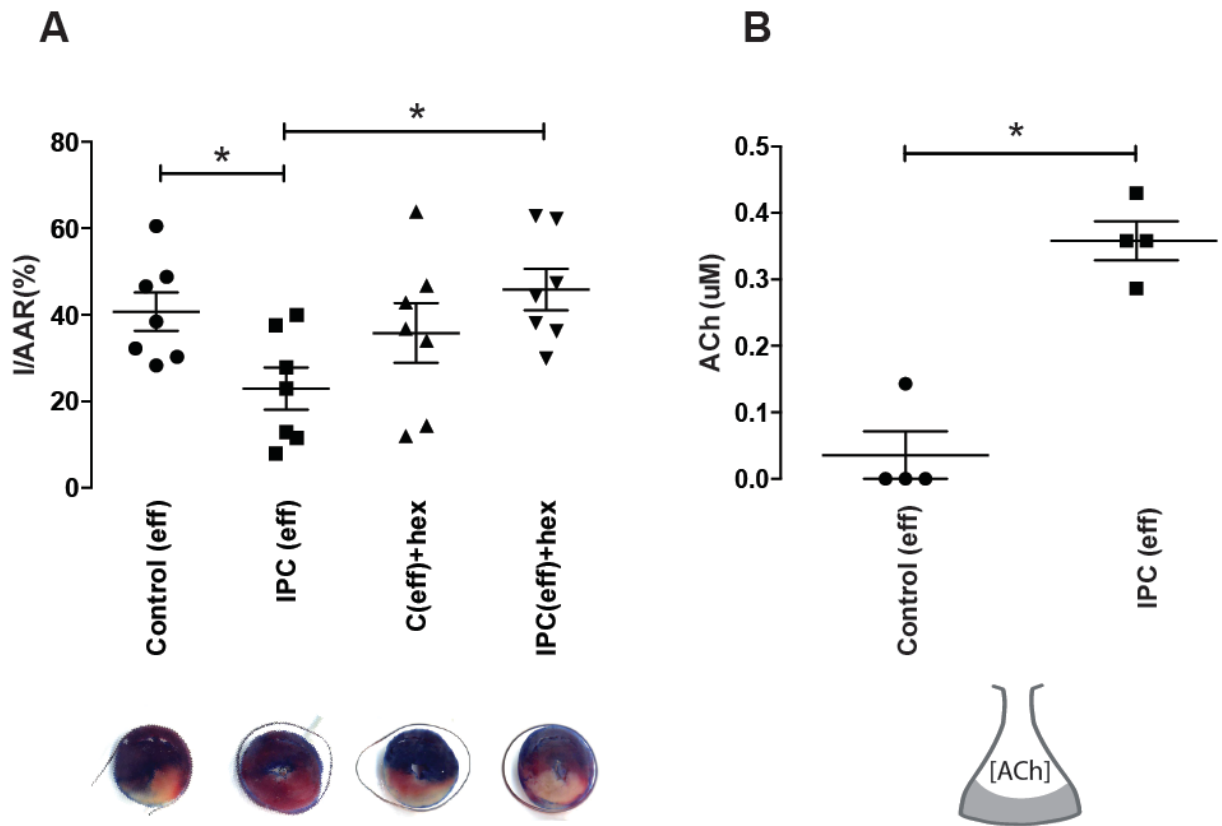
#### 6.4.2 Study 2: Factors released following classical IPC require intrinsic cardiac nerves to induce protection

Effluent collected from hearts following classic IPC induced significant protection when perfused through a second or naïve isolated rat heart prior to acute IRI (IS/ARR =  $19 \pm 2$ ,  $p < 0.05$  vs control IS/AAR =  $46 \pm 6\%$ ). Pre-treatment of the naïve recipient heart with the nicotinic antagonist, hexamethonium, abrogate the protection offered by IPC effluent (IS/AAR =  $46 \pm 5\%$ ,  $p < 0.05$  vs IPC effluent) (Fig. 3A).

A large release of ACh following IPC was observed in these isolated perfused rat hearts, with a 10-fold increase in the concentration relative to control effluent ( $IPC_{eff} = 0.36 \pm 0.03 \mu M$  vs  $C_{eff} = 0.04 \pm 0.04 \mu M$ ,  $n = 4$ ,  $p < 0.001$ ) (Fig. 3B). Three of the four control effluent samples did not contain any detectable ACh.

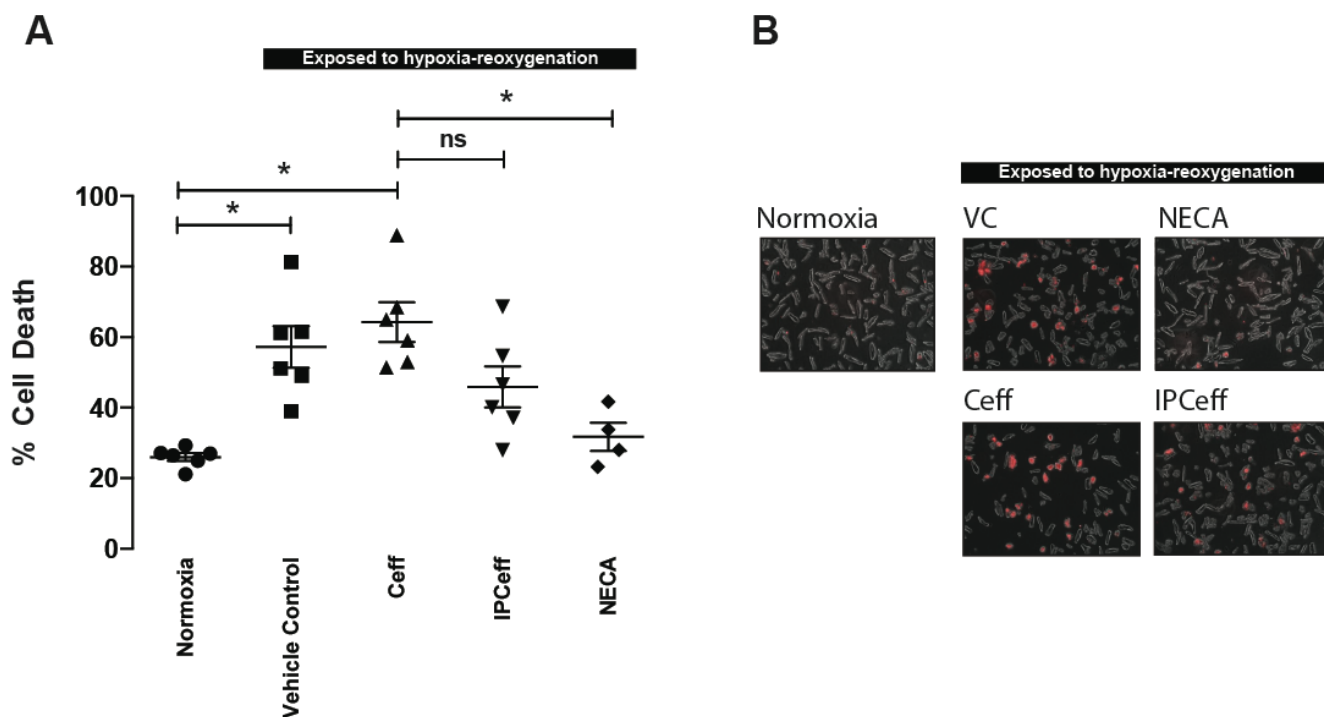
*Classic IPC effluent appears not to protect isolated cardiomyocytes from simulated IRI*

Cells that were maintained under normoxic conditions throughout the experiment exhibited  $27 \pm 2\%$  cell death (Fig. 4). In cells that underwent simulated IR, this was increased to  $57 \pm 6\%$  and  $64 \pm 6\%$  after pre-treatment with the vehicle control and control effluent, respectively ( $p < 0.001$  vs normoxic in both cases). Treating the cells with IPC effluent did not significantly reduce cell death ( $45 \pm 6\%$ ,  $p=0.09$  vs  $C_{eff}$ ). The adenosine A2b agonist NECA (used as a +ve control) significantly reduced cell death to  $32 \pm 4\%$  ( $p < 0.01$  vs vector control and  $C_{eff}$ ) (Fig. 4).



**Figure 6-3: IPC effluent protects a naïve heart from ischaemia-reperfusion injury.** (A) effluent collected following IPC significantly protected a naïve isolated rat heart via a hexamethonium-sensitive mechanism ( $n=7-8$  per group,  $* = p<0.05$  vs control effluent); (C) The concentration of ACh in coronary effluent increases 10-fold following IPC ( $n=4$  per group,  $* = p<0.05$  vs Control effluent). Data presented as mean  $\pm$  SEM.





**Figure 6-4: IPC effluent does not protect isolated cardiomyocytes from simulated ischaemia-reperfusion injury.** (A) Isolated rat cardiomyocytes were not protected from hypoxia reoxygenation injury by prior exposure to IPC effluent. The adenosine A2B agonist, NECA, was able to reduce cell death significantly ( $n=6$  in all groups except  $n=4$  for NECA,  $* = p<0.05$  vs normoxia); (B) representative images of isolated cardiomyocytes subjected to the different protocols, the red staining indicate dead cells. Data presented as mean $\pm$ SEM.

## 6.5 Discussion

These results are the first indication of a neural pathway in the mechanism of classical ischaemic preconditioning. We demonstrated an important role for nicotinic and acetylcholine receptors, suggesting that intrinsic cardiac ganglia remain active in the isolated heart preparation and are important in conveying the protective message. Acetylcholine is released from the heart following IPC, perhaps from parasympathetic post-ganglionic nerve endings in the ventricles, and induces protection via an atropine-sensitive mechanism. The lack of total abolition of protection, in the presence of ganglionic or muscarinic antagonism, is likely due to the fact that several factors contribute to the IPC mechanism in the isolated heart model [86]. Moreover, coronary effluent collected following IPC was able to protect a naïve isolated heart, but not isolated cardiomyocytes, from IRI. The protection in naïve hearts was abrogated by hexamethonium, highlighting the importance of intrinsic cardiac ganglia in the mechanism of IPC. We therefore propose that IPC is governed, in part, via a neuro-humoral pathway; a factor released following IPC activates intrinsic cardiac ganglia, leading to release of ACh from parasympathetic post-ganglionic nerve endings in the ventricles, thus inducing cardioprotection via activation of muscarinic receptors. These data are, to some extent, additive to our previous study, where we proved an important role for intrinsic cardiac ganglia in remote ischaemic conditioning. Whilst a neural pathway has been well validated in RIC, this is the first to imply a similar intrinsic cardiac neural pathway in IPC. Thus, there appear to be more similarities between the mechanisms of classical (direct) and remote ischaemic conditioning than were previously apparent [24, 278].

### *Intrinsic cardiac ganglia in the isolated heart*

Intrinsic cardiac ganglia are widely distributed in the myocardium and not only relay central efferent pre-ganglionic information, but also are able to process sensory afferent information from the myocardium and control efferent post-ganglionic firing [26, 230]. Moreover, several anatomical and functional studies have indicated a significant presence of vagal neurons in

the ventricles, in addition to sympathetic and local circuit neurons [15]. The intrinsic cardiac nervous system is therefore able to control cardiac indices on a beat-to-beat basis, in the absence of input from the central nervous system [16].

Activation of sensory afferent nerves in the Langendorff heart has previously been demonstrated to induce cardioprotection. Perfusion with capsaicin, a known activator of C-fibre afferents, induced early and delayed protection against ischaemia-reperfusion injury in isolated Langendorff rat hearts [406]. In our study, ganglionic antagonism abrogated the protection afforded by IPC, suggesting sensory afferent activation occurs following IPC and is important in conferring the cardioprotection. We further demonstrated a 10-fold increase in the ACh concentration in perfusate collected following IPC. This suggests activation of post-ganglionic parasympathetic neurones from the intrinsic ganglia. Indeed, muscarinic antagonism, using atropine, abolished IPC-induced cardioprotection. The parasympathetic nervous system has a well defined cardioprotective effect [174, 242, 243, 322], and recently emerged as a key mediator of the cardioprotection afforded by “remote” ischaemic conditioning (RIC) [25, 243, 278]. Indeed, a recent study demonstrated that increased parasympathetic tone ameliorated the functional and structural remodelling of the intrinsic cardiac nervous system following myocardial infarction [27]. Thus, we propose that there exist similarities between the mechanism of remote and classical ischaemic conditioning; an intrinsic neural reflex loop in response to the brief ischaemia of IPC, which activates cardiac ganglia and increases post-ganglionic vagal tone, leading to release of ACh in the ventricles.

#### *A contentious role for acetylcholine in IPC*

IPC is triggered via release of several small molecules, and their subsequent receptor activation in the myocardium. This was first demonstrated by Liu et al, who showed that pre-treatment with an adenosine receptor antagonist abrogated IPC in rabbit hearts [226]. Exogenous adenosine, perfused through the heart prior to infarction, mimicked the cardioprotection of IPC.

These data suggested endogenous release of adenosine occurs in response to IPC, which protects the myocardium. Two other small molecules, opioids [233] and bradykinin [357], were found in subsequent studies to be important in the mechanism of IPC, via activation of their receptor. These appear to be connected via a common intracellular cytoprotective signalling pathway [267], which centres on activation of protein kinase C (PKC) [86]. Coronary effluent collected following IPC can protect a naïve isolated heart from infarction [43, 75], supporting the theory of a humoral trigger for IPC. Indeed, adenosine is released into the effluent following IPC, and confers protection to the naïve heart via crosstalk with opioid receptors [74, 215]. Bradykinin is not involved in this setting due to the requirement for circulating kininogens in the blood, not present in the isolated buffer-perfused model [105].

The role for acetylcholine in IPC, however, is more contentious. While several studies from Krieg et al. demonstrated exogenous ACh could induce cardioprotection in the Langendorff model, the same group discounts its involvement in the mechanism of IPC [200–202]. However, two studies from Kawada et al. demonstrated brief, 5 min ischaemia in *in vivo* rabbit and cat models induces interstitial release of ACh in the ventricles [176, 177]. Data from our study confirm those of Kawada et al, with a significant release of ACh observed in coronary effluent following IPC. Given this observation, we hypothesised that isolated cardiomyocytes would be protected from simulated IRI following exposure to coronary effluent. Presumably, if ACh was mediating protection here, it would act directly on muscarinic receptors on the cardiomyocytes in the naïve heart. However, IPC effluent was not able to significantly reduce cell death in isolated cardiomyocytes subjected to hypoxia-reoxygenation. A small reduction in cell death is observed, likely due to the presence of several trigger factors in the effluent [74, 215], however this was not statistically significant. This is perhaps due to dilution of factors released from the myocardium in the effluent following IPC, such that the concentration in the isolated cells would be insufficient for cardioprotection. However, we did not investigate whether exogenous ACh of the same concentration could induce cardioprotection in isolated cardiomyocytes or Langendorff models.

Finally, coronary effluent collected following IPC induced powerful cardioprotection when perfused through naïve isolated rat hearts, via a mechanism also sensitive to hexamethonium. This experiment confirms the key point to the study; factors released following IPC require intrinsic cardiac ganglia to induce cardioprotection. The neural and humoral components to IPC, therefore, are co-dependent.

*Is there a common trigger for classical and remote ischaemic conditioning?*

Our data suggest that both classical and remote ischaemic conditioning (RIC) may share a common trigger pathway; i.e. local release of an autocoid, activation of sensory afferent nerves and subsequent recruitment of the intrinsic cardiac nervous system. RIC is induced via the same principle as classical IPC, brief cycles of ischaemia, however applied to a region remote from the heart [35]. The trigger for RIC is thought to be local release of an autocoid, such as adenosine, which activates sensory afferent nerves communicating the protective message away from the conditioned limb. Indeed, a small injection of adenosine into the femoral artery is sufficient to induce cardioprotection [331], as is activation of C-fibre sensory afferents by capsaicin [290] or transcutaneous electrical nerve stimulation [248]. Moreover, our study has demonstrated the importance of intrinsic cardiac ganglia in IPC, which necessitates prior sensory nerve activation in the heart. Classical IPC is known to involve release of adenosine and calcitonin gene related peptide [215, 226], both of which are known to activate sensory afferent nerves. Perhaps, therefore, these are the trigger for this aspect to IPC. The trigger for both RIC and classical IPC appear to share important similarities, with both neural and humoral components [278].

A recent meta-analysis revealed that ischaemic preconditioning had variable efficacy in mammalian species; namely, IPC was more effective in rodents relative to non-rodents [368]. Myocardial autonomic innervation is known differ according to the species' size [310], however it is not clear whether these differences relate to the species-specific effect of IPC. Finally, with respect to remote ischaemic conditioning, although there appear to be

differences in the signalling cascades important for cardioprotection between species [324], a recent meta-analysis revealed no difference in the efficacy of cardioprotection relative to species [45].

### *Study limitations*

The key limitation of the study lies in the specificity of hexamethonium to neuronal nicotinic acetylcholine receptors. Hexamethonium is widely used within the literature as a ganglionic blocker, being a neuronal nicotinic acetylcholine receptor antagonist. Although it has some affinity to muscarinic M2 receptors, this becomes negligible at concentrations below 100  $\mu\text{M}$  [88], and it has an  $\text{IC}_{50}$  of 11  $\mu\text{M}$  at nAChRs [109]. Thus, for the purposes of this study 50  $\mu\text{M}$  was used in order to achieve high specificity to nicotinic receptors, but the potential may remain for non-specific effects. The second issue relates to the spatial expression of nicotinic receptors within the myocardium. Although nicotinic receptors are largely limited to the intrinsic cardiac ganglia, there is evidence for expression of the receptors on the myocytes [87]. Thus, there is a small possibility that hexamethonium is exerting its effect through non-ganglionic action.

Secondly, these data do not fully ascertain the role of ACh within the effluent. An important additive experiment would be to treat a naïve-isolated heart with acetylcholine at the same level observed within the effluent. In addition, it is possible that stimulation of isolated cardiomyocytes with effluent for longer than 10-min could reveal cardioprotection.

## **6.6 Summary**

This is the first study to implicate intrinsic cardiac ganglia in the mechanism of classical ischaemic conditioning. We propose that IPC activates an intrinsic cardiac neural reflex and is an important part of the cardioprotective mechanism. This is a significant finding for several reasons. The Langendorff perfused heart was traditionally thought of as a denervated preparation, however clearly this is not true given the current data, in addition to our recent publication [278]. Moreover, these data add to the paradigm that IPC

is a receptor-mediated phenomenon. However, there seems to be an added layer of complexity, with the intrinsic ganglia responsible for conferring a portion of the cardioprotection. This is of importance given the issue of co-morbidities in the clinical setting. For example, diabetes has been well documented to decrease the efficacy of IPC [372]; perhaps this could be explained by the peripheral sensory neuropathy that occurs as the disease progresses [164]. Whether the peripheral sensory nerve activation induced via remote ischaemic conditioning is comparable to that of classical ischaemic conditioning is not clear. Further work is necessary to ascertain the exact nature of the involvement of intrinsic ganglia in this setting.

## **Chapter 7 STIMULATING THE EAR TO PROTECT THE HEART: TRANSCUTANEOUS VAGUS NERVE STIMULATION IS A NOVEL CARDIOPROTECTIVE INTERVENTION**

### **7.1 Introduction**

There remains a clinical need for interventions that limit the extent of myocardial ischaemia-reperfusion injury. One such intervention, remote ischaemic conditioning (RIC), has proven popular in translational research due to its non-invasive nature and promising pre-clinical data [35, 276, 283]. Subjecting the upper or lower limb to brief periods of ischaemia and reperfusion can communicate significant protection to the myocardium from an injurious ischaemic insult. Clinical trials have investigated the efficacy of RIC in limiting the injury associated with coronary artery disease in several settings, including ST-elevation myocardial infarction (STEMI), and coronary artery bypass surgery (CABG). Whilst a majority have demonstrated cardioprotection, particularly in patients suffering from STEMI [41, 68, 294, 325, 370], two recent large-scale trials failed to demonstrate improved outcome in patients undergoing coronary artery bypass surgery [122, 249]. Thus, investigation into the basic mechanisms underlying RIC has gained new importance; not only to explain the current lack of translation, but also to offer new opportunities by which the cardioprotective reflex can be accessed.

The key process that requires further laboratory investigation is the communication of the protective message from the conditioned limb to the myocardium. The focal point of this communication in the literature is release of a blood-borne humoral factor. Shimizu et al demonstrated that a plasma dialysate generated following RIC in human volunteers was able to protect a naïve isolated rabbit heart [320]. This highlighted the remarkably conserved and stable nature of the humoral factor. Several subsequent studies demonstrated that release of the humoral mediator is dependent on intact sensory innervation to the conditioned limb [248, 289, 290, 331]. Moreover, increased vagal tone has been proven essential in the communication of RIC from the conditioned limb to the myocardium [79, 243, 278]. Genetic silencing



of vagal preganglionic neurones, in the dorsal vagal motor nucleus (DMVN), abrogated in vivo RIC in a rat model of acute myocardial infarction. Conversely, stimulation of the DMVN via optogenetics was sufficient to induce cardioprotection in vivo [243]. In addition, a recent paper from the same group suggested non-cardiac vagal innervation is fundamental to the RIC mechanism [242]; section of the posterior gastric branch of the vagus nerve abrogated RIC-mediated cardioprotection. Finally, we have recently demonstrated that bilateral cervical vagotomy prior to RIC abrogates release of the humoral mediator [278]. This suggests a complex neuro-humoral mechanism of RIC communication, initiated with sensory afferent activation in the conditioned limb leading to increased vagal tone and subsequent release of a humoral mediator, perhaps from the gut [25].

There may, therefore, be an opportunity to bypass the RIC cuff and stimulate the vagus nerve directly to achieve the same cardioprotective endpoint. Several pre-clinical studies have demonstrated as much, with vagus nerve stimulation inducing powerful cardioprotection in animal models of acute ischaemia-reperfusion injury [174, 347]. Within the clinic, VNS is difficult to achieve in the setting of acute myocardial infarction (AMI) given the invasive procedure required to fit the stimulating device. Emerging data, however, have demonstrated that the tragus of the ear can be stimulated electrically to increase systemic vagal tone [62]. Indeed, the vagus nerve provides a large portion of the sensory innervation to the ear, in particular to the tragus and inner concha (Fig. 7-1) [32, 275]. Electrical stimulation of these regions, known as transcutaneous vagus nerve stimulation (tVNS), has been demonstrated to produce cognitive, behavioural and cardiovascular effects similar to that of direct vagus nerve stimulation [20, 299, 393]. Indeed, a recent clinical study demonstrated that sensory nerves in the tragus project to the nucleus tractus solitarius (NTS), a central autonomic centre, and thus are consistent with classical vagal projections [96]. Therefore, tVNS is emerging as a viable non-invasive mechanism to influence systemic vagal tone. This chapter investigates whether tVNS is a viable novel cardioprotective intervention.

## **7.2 Research aims and objectives**

There were two main objectives in this chapter:

1. Investigate efficacy of transcutaneous vagus nerve stimulation in releasing cardioprotective blood-borne factors in healthy human volunteers;
2. Investigate transcutaneous vagus nerve stimulation in an *in vivo* rat model of myocardial ischaemia-reperfusion injury.

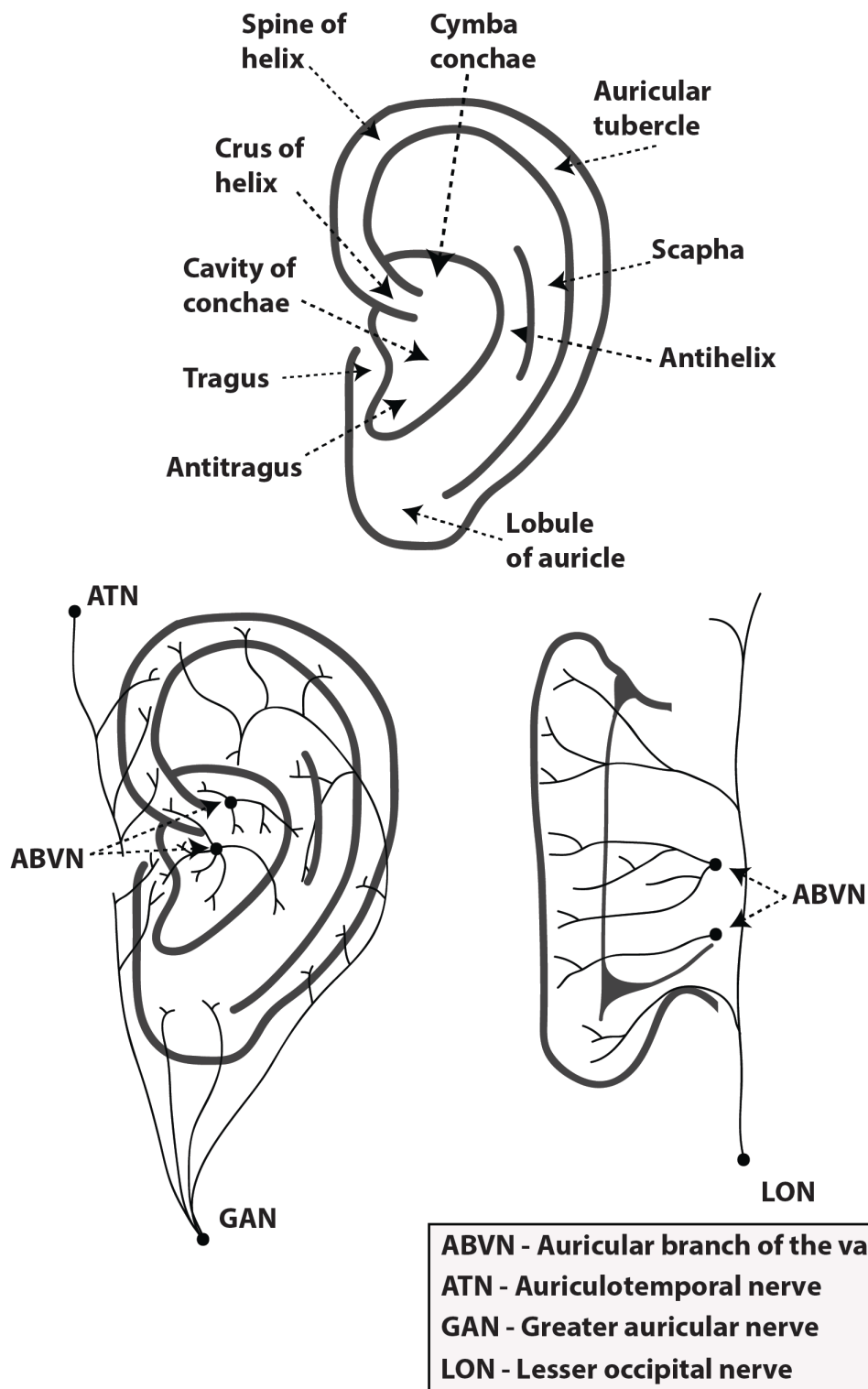
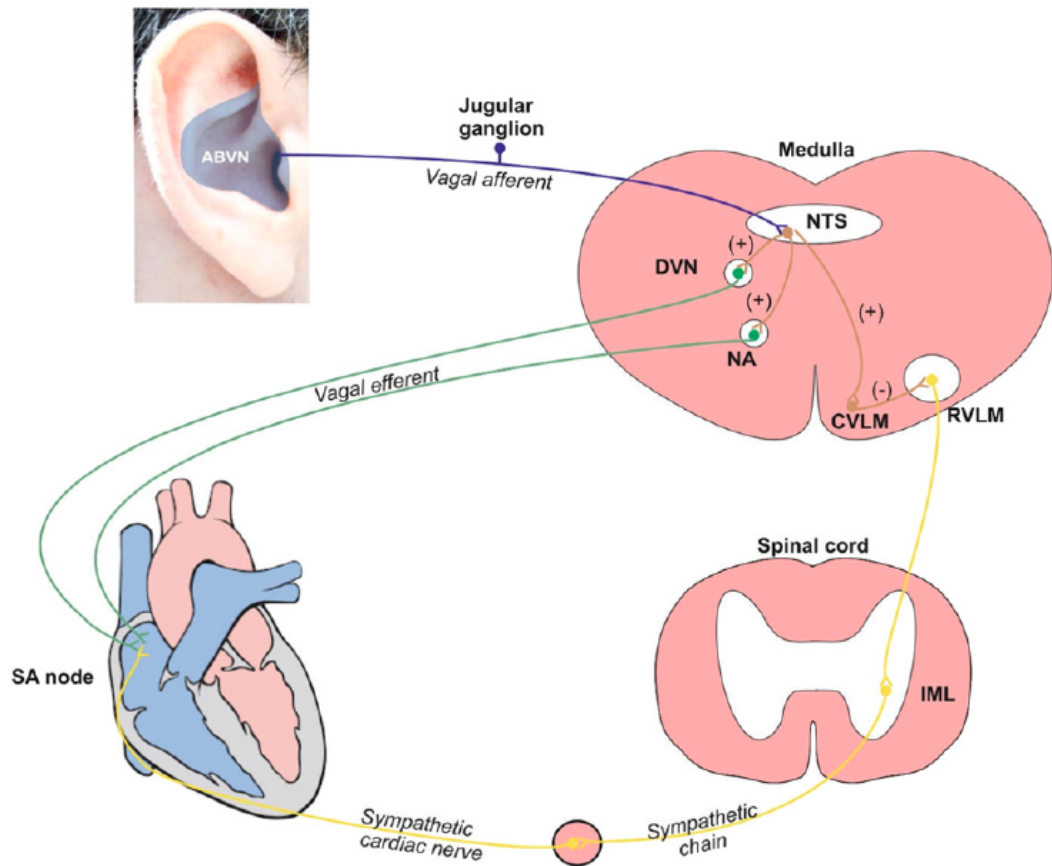


Figure 7-1. Schematic displaying outer ear anatomy with general sensory innervation (figure adapted from that shown in [275]).



**Figure 7-2. Proposed pathway by which transcutaneous vagus nerve stimulation elicits its effect on the myocardium.** Figure taken from [254]. Afferent projections from the auricular branch of the vagus nerve synapse with cell bodies in the nucleus of the solitary tract (NTS). Projections from the NTS can control both sympathetic and parasympathetic outflow to the heart. DVN=Dorsal vagal nucleus, NA=Nucleus ambiguous, CVLM/RVLM=Caudal/Rostral ventrolateral medulla, IML=intermediolateral nucleus.

### **7.3 Aim 1: transcutaneous vagus nerve stimulation in healthy human volunteers**

Below describes a collaborative project with Prof Jim Deuchars' laboratory in the University of Leeds. The study was conceived and designed by Prof Derek Hausenloy, Dr Nephtali Marina and myself. The first part of the study, involving human volunteers, was carried out by Aaron Murray at the University of Leeds. The second part of the study, involving the dialysate model described in section 3.5, was performed at the Hatter Cardiovascular Institute by myself. The manuscript is currently under review with JACC: Basic to Translational Science

**Pickard JM, Murray A, Marina-Gonzalez N, Yellon DM, Deuchars SA, Deuchars J, Hausenloy DJ** *Transcutaneous vague nerve stimulation induces release of a dialysable cardioprotective factor in healthy volunteers.* (under review)

#### **7.3.1 Background**

Sensory innervation of the ear is supplied by branches of three cranial nerves; the vagus, trigeminal and facial (see Fig. 7-1). A branch of the vagus nerve, called the auricular branch of the vagus nerve (ABVN), innervates the cymba concha, tragus, antihelix and cavity of concha [275]. The ABVN is a remnant of the embryonic nerve supplying the first branchial arch, and arises from the superior jugular ganglion of the vagus nerve [115]. In rats and humans, afferent projections from the ABVN have been demonstrated to terminate at the nucleus of the solitary tract (NTS), dorsal raphe, locus coeruleus and amygdala [96, 135]. Of particular interest with respect to a cardiovascular effect is the NTS, which is known to control efferent parasympathetic tone from the dorsal vagal motor nucleus and the nucleus ambiguus. The NTS is also able to communicate with sympathetic centres in the ventrolateral medulla. Clancy et al. demonstrated that transcutaneous vagus nerve stimulation (tVNS) in healthy human volunteers improved heart rate variability, indicating parasympathetic predominance [62]. In the same study, a decrease in sympathetic nerve activity was measured via

microneurography following tVNS [62]. These effects are consistent with a neural pathway for tVNS, proposed by Murray et al. in a recent review (see Fig. 7-2 and [254]).

Transcutaneous vagus nerve stimulation has therefore emerged as a novel mechanism to non-invasively increase systemic vagal tone. Indeed, given the wide-ranging innervation of the vagus nerve, this intervention is being investigated in the treatment of several pathologies, including epilepsy, depression and cardiovascular disease.

Given the importance of the vagus nerve in the release of the blood-borne humoral mediator of RIC [278], we hypothesised that non-invasive vagus nerve stimulation might mimic RIC and induce release of a similar cardioprotective factor. We performed a study using healthy human volunteers, in collaboration with Prof Jim Deuchars University of Leeds. The Deuchars research group has carried out significant work on transcutaneous vagus nerve stimulation [62], and have the requisite facilities and ethical approval to perform a randomised human trial using tVNS.

### **7.3.2 Methods**

#### *Human tVNS trial design – University of Leeds*

Ten healthy volunteers were recruited to the study (see Table 1 for participant details). Each volunteer was required to attend the Deuchars' laboratory at the University of Leeds on two separate occasions spaced at least 7 days apart. On the first visit, each volunteer was randomised to receive either remote ischaemic conditioning (RIC) (4 cycles of 5 min inflation to 200mmHg and 5 min deflation of cuff around upper limb), or 40 minutes of non-invasive low-level electrical stimulation applied to the tragus of the ear via an auricular electrode clip connected to a transcutaneous electrical nerve stimulation (TENS) machine. The tVNS stimulation protocol consisted of 200µs pulse width at 30Hz, with the amplitude varied (10-50mA) depending of sensory threshold of the subject. The volunteer would return at least 1 week later and receive the alternate intervention to the first visit. For both of

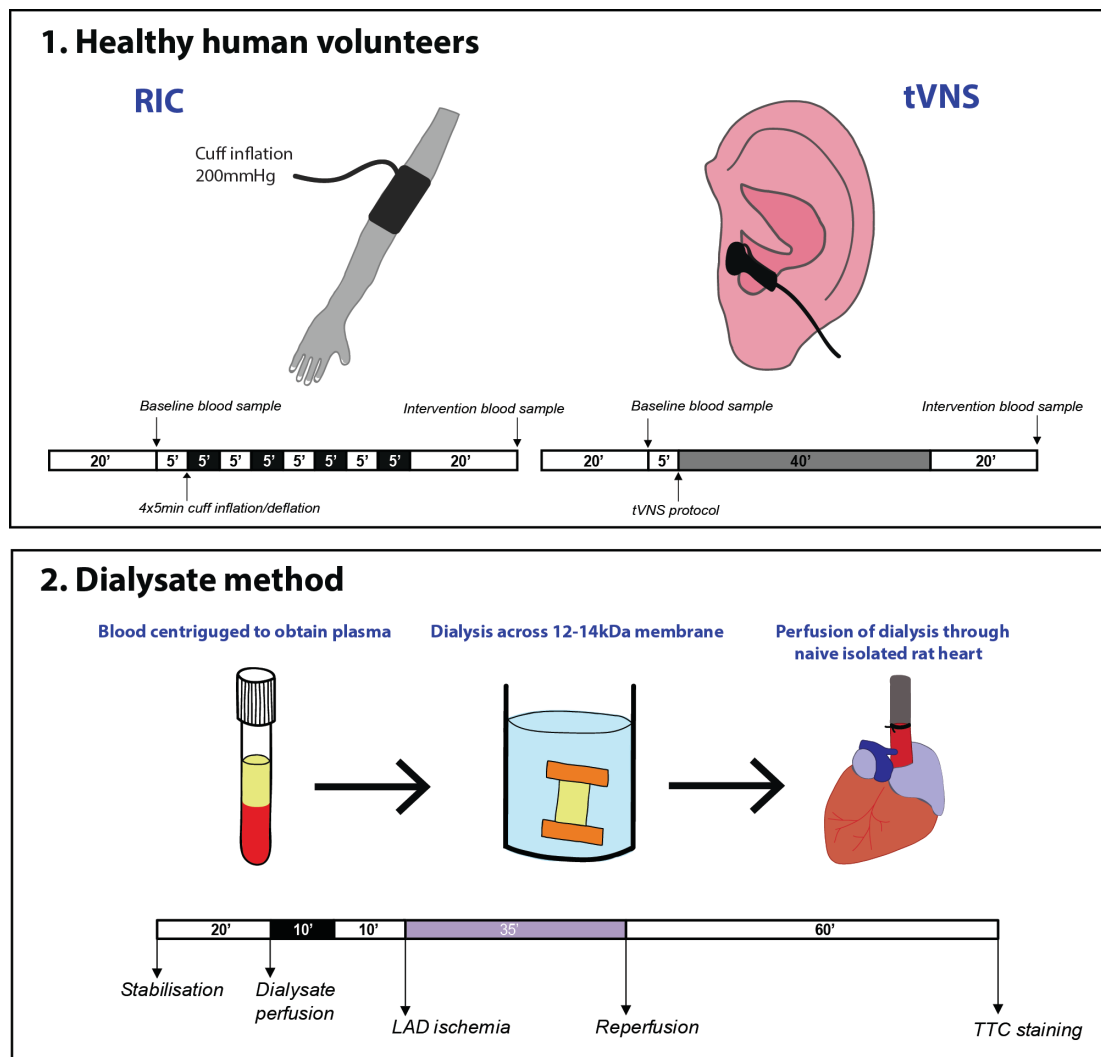
the above protocols, a baseline blood sample was taken from an antecubital vein into a heparinised vial before the intervention is applied and a second blood sample will be taken after RIPC or tVNS (Fig. 1). In addition, three sets of data (ECG, blood pressure and respiration) were recorded from research participants during their first visit to the lab: (1) a 10 minute baseline (control) period before the intervention/stimulation was applied, (2) the 40 minute intervention period during which either tVNS or RIPC is applied and (3) a 10 minute recovery period after the intervention. When participants were receiving RIC, data were derived from the 5min episodes of reperfusion. The participants reported mild to moderate discomfort during the ischaemic episodes, this may have confounded the HR measurements. The ECG recordings were analysed using Spike2 software to calculate the time interval between each individual heart. Changes in heart rate are separated into high and low frequencies. This technique can then be used to determine heart rate variability (HRV) - a measure of how much variation there is in the time intervals between heartbeats. An improved HRV is associated with increased activity in the parasympathetic nervous system.

Blood samples were immediately centrifuged at 1600 g for 20 min at 21°C to obtain plasma, followed by 10,000g for 30min at 21°C to obtain 4mls of platelet-free plasma. This was then frozen at -80°C. After collection of all the plasma samples, these were sent in batch to the Hatter Cardiovascular Institute.

Participant	Gender (M/F)	Age	Height (cm)	Weight (kg)	BMI
UCL1	M	48	172	68	22.9
UCL2	F	35	158	45	18
UCL3	M	26	174	73	24.1
UCL4	M	24	183	69	20.6
UCL5	F	49	164	60	22.3
UCL6	F	29	167	59	21.1
UCL7	M	23	180	79	24.3
UCL8	F	23	174	71	23.4
UCL9	F	30	163	57	21.4
UCL10	M	22	181	87	26.5

**Table 7-1. General characteristics of healthy participants in the study.** *The table displays the gender, height (cm), age and weight of each participant.*





**Figure 7-3. Schematic displaying experimental protocols.**

*Dialysate preparation and perfusion through naïve isolated rat hearts – Hatter Cardiovascular Institute*

The dialysate model has been previously described in this thesis (section 3.5). Briefly, plasma samples were thawed and dialysed across a 12-14kDa membrane into 200mls of modified KHB for 24hrs at 4°C. Dialysate was then perfused through a naïve isolated rat heart for 10min prior to a 35min ischaemia and 60min reperfusion. Infarct size was analysed using triphenyltetrazolium chloride staining (section 2.2.5).

*Statistical analysis*

For the ECG and respiration measurements, data were first analysed for normality using the Shapiro-Wilk test. One-way repeated measures ANOVA was used to assess changes in each dataset alone over time. Friedman's test and Wilcoxon signed ranks test used for data that was not normally distributed. For the infarct analysis, data groups were first analysed for normality using the Kolmogorov-Smirnov test. Both tVNS and RIC groups were analysed relative to their respective baseline dialysate groups using a paired student's t-test. Haemodynamic data from the Langendorff experiments were analysed using a 2-way repeated measures ANOVA with Bonferroni's post-test. All data are presented as mean  $\pm$  standard error of the mean (SEM). Data groups were classed as significantly different with a p value less than 0.05. Notation of significance was as follows: \*=p<0.05, \*\*=p<0.01 and \*\*\*=p<0.001. Analysis was exclusively performed using GraphPad Prism version 5 for Windows (CA, USA).

### 7.3.3 Results

#### *Effect of tVNS/RIC on ECG and respiration rate data*

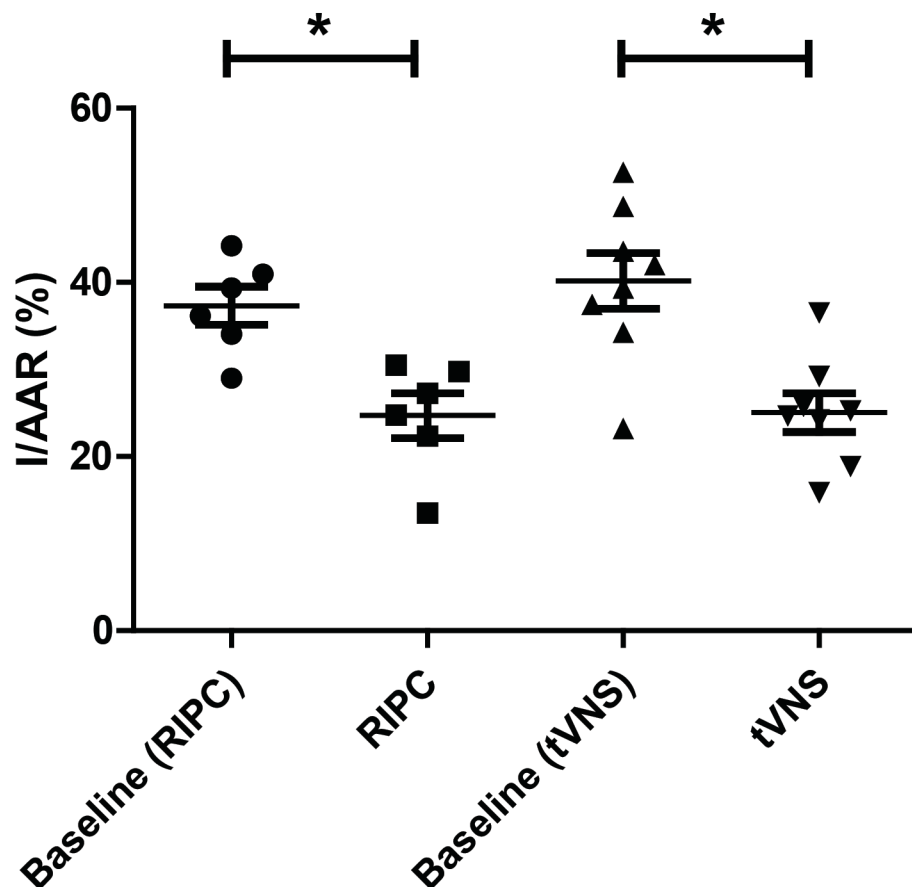
Figure 2 displays the heart rate (HR) and heart rate variability (HRV) recorded for each patient whilst receiving either RIC or tVNS. No significant differences were observed in HRV between both interventions over time. No differences were observed in HRV during either tVNS or RIC alone over time. A significant change in HR over time was observed during tVNS, but not during RIC. However, no significant differences were observed between both intervention groups over time. Respiration rate was not significantly altered during either intervention over time.

	Baseline	10min	20min	30min	40min	Recovery
<b>LF/HF ratio</b>						
<i>tVNS</i>	1.13(0.23)	0.84(0.14)	0.93(0.14)	1.12(0.24)	1.10(0.25)	1.15(0.23)
<i>RIC</i>	0.84(0.17)	0.83(0.19)	0.87(0.17)	1.28(0.23)	1.01(0.16)	0.99(0.23)
<b>Heart Rate</b>						
<i>tVNS</i>	61.6(1.2)	60.3(1.6)	59.8(1.5)	59.9(1.5)	61.8(1.3)	62.9(1.2)
<i>RIC</i>	60.9(2.5)	60(2.0)	59(2.3)	58.5(2.2)	59.6(2.0)	60.9(2.2)
<b>Respiration Rate</b>						
<i>tVNS</i>	16.7(0.6)	16.6(0.6)	16.5(0.5)	16.6(0.7)	16.4(0.8)	17(0.6)
<i>RIC</i>	16(0.6)	16.6(0.7)	16.4(0.7)	15.7(0.9)	16(0.9)	15.8(0.7)

**Table 7-2. ECG and respiration rate data collected over the course of each intervention.** A baseline measurement was taken prior to each intervention. Measurements were taken at 10min intervals during both RIC and tVNS. A final measurement was taken following a 20min recovery period. Data presented as mean(SEM). Heart rate in patients undergoing tVNS was found to significantly change over time ( $p > 0.05$ ). All other groups displayed no statistical significance.

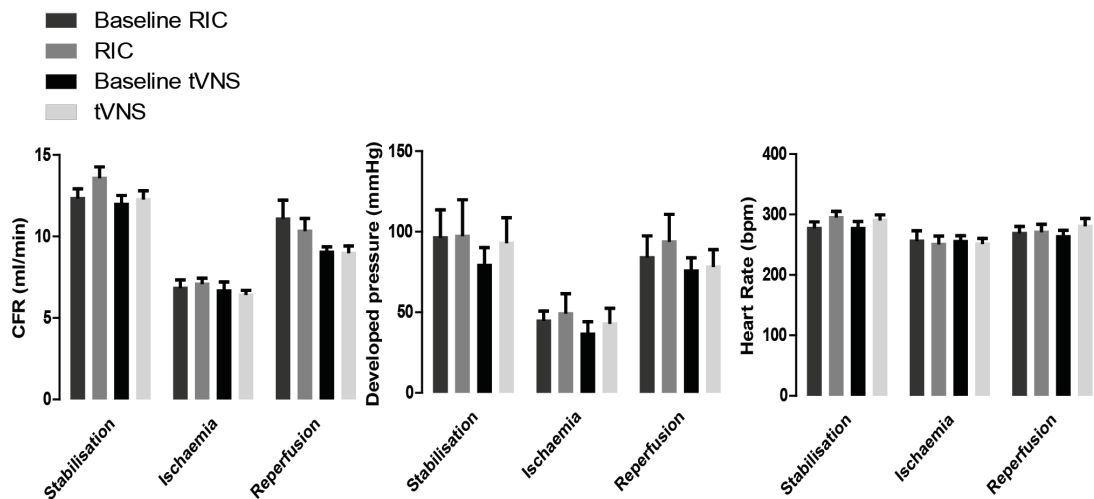
*tVNS dialysate is equally as protective as RIC dialysate*

Although 10 participants were recruited from the study, blood was only sampled from 8 due to 2 of the subjects experiencing a collapsed vein. Two of the plasma samples in the RIC group were not usable, leaving 6 usable samples. Dialysate prepared following RIC in human volunteers significantly protected the naïve isolated rat heart from IRI, relative to baseline dialysate. Interestingly, tVNS dialysate also induced significant cardioprotection to the naïve rat heart with respect to baseline dialysate. The protection offered by tVNS dialysate was equal to that of RIC dialysate.



**Figure 7-4. Dialysate prepared following tVNS protects a naïve heart to a similar level to that observed with RIC dialysate.** Chart displaying infarct size of naïve perfused rat hearts that received human dialysate prepared following either RIC or tVNS. All hearts received 35min left anterior descending coronary artery ischaemia and 60min reperfusion. Data presented as mean  $\pm$  SEM.

Figure 3 displays the haemodynamic measurements taken during the Langendorff experiments. No significant differences were observed between the groups in coronary flow rate, left ventricular developed pressure or heart rate over the ischaemia-reperfusion protocol.



**Figure 7-5. Haemodynamic data from the naïve hearts that received dialysate.** Measurements of coronary flow rate, left ventricular developed pressure and heart rate in the naïve perfused rat hearts that received dialysate across the ischaemia-reperfusion injury protocol. Data presented as mean  $\pm$  SEM. No statistical significance was observed following a repeated measures ANOVA.

#### 7.3.4 Discussion

This study is, to our knowledge, the first to demonstrate the cardioprotective potential of tVNS. Although we do not measure a direct protective benefit in the human volunteer, the indirect endpoint indicates that a humoral cardioprotective factor is released into the blood following tVNS. Similarly, we demonstrate that RIC in human volunteers produces a dialysate that can protect a naïve rat heart. Although this has been observed in several other studies, it reinforces the interesting observation that the humoral factor is highly conserved and thus affords this cross-species protection. Given RIC is known to require vagus nerve activation as part of the mechanism, tVNS could be a novel intervention to activate similar cardioprotective pathways.

Growing evidence suggests that vagal afferents in the outer ear are linked to central vagal centres. Stimulation of the concha, which contains only vagal afferents, resulted in activation of the nucleus of the solitary tract (NTS) and other projections consistent with vagus nerve activation [96, 135]. Sensory innervation to the tragus, however, is supplied by the trigeminal and vagus nerves in roughly equal proportions [275]. Therefore, it is plausible that the beneficial effects of tragus stimulation come from trigeminal, as well as vagal, afferent activation. Recent evidence, however, strongly indicate a systemic vagal effect following tVNS. Clancy et al demonstrated an increase in heart rate variability (HRV) and decrease in sympathetic nerve activity following tVNS to the tragus [62]. Thus, given the pre-clinical data regarding the vagus nerve and RIC, in combination with the study above, it is likely that the cardioprotective effect of tVNS is due to vagus nerve activation in the tragus.

There was a small, but non-significant, decrease in LF/HF ratio during tVNS. This indicates an increase in heart rate variability, and vagal predominance. Although this was not statistically significant, the absolute decrease observed was similar to that measured by Clancy et al. (n=48), where a significant decrease in LF/HF was observed. The lack of statistical significance with our data is perhaps an effect of the natural variability in vagal tone between participants, thus increasing the numbers may reveal a significant effect.

Several recent studies, including one from this group, demonstrated that release of the humoral blood-borne factor following RIC is dependent on an intact vagus nerve [25, 242, 278]. Sectioning of the vagus nerve at the cervical level abrogated release of the humoral cardioprotective factor following RIC. Moreover, section of the vagus nerve below the diaphragm abrogated the cardioprotection induced by RIC in an *in vivo* rat model of myocardial infarction. This suggested that non-cardiac vagal innervation was important in the mechanism of RIC, which acted to release a humoral blood-borne cardioprotective factor. This factor has since been suggested to be glucagon-like peptide 1 (GLP-1). The current paradigm therefore suggests that RIC increases vagal tone to an organ below the diaphragm, likely the gut, triggering release of GLP-1 into the blood, which then moves to the heart and induces protection. Our data provides the first evidence that the increase vagal tone achieved following RIC can also be recruited by tVNS; both interventions produce a dialysate that is equally protective to a naïve rat heart. Finally, we did not observe a correlation between HRV following tVNS and infarct size ( $R^2 = 0.014$ ). This suggests that the cardiovascular effect of tVNS is not responsible for the cardioprotection, in line with the recent literature described above. Although not proven in the current study, our suggestion is that RIC and tVNS share key mechanistic similarities.

There is great deal of heterogeneity in the application of tVNS in the literature, both in terms of site and parameters of stimulation on the ear [254]. However, there are a growing number of promising trials demonstrating a benefit in several cardiac pathologies. Two pioneering studies in the field were performed on patients undergoing coronary artery bypass surgery [397, 398]. Electrical stimulation to the cymba concha, via acupuncture needles attached to both ears, was applied for 15min per day for 10 days following the operation. Patients who received stimulation had a better outcome following surgery, as measured by improved left ventricular ejection fraction and reduced incidence of angina at rest. A more recent study stimulated the internal surface of the auricle for 10 days in patients suffering from CAD [279]. The incidence of angina attacks significantly decreased and this was associated with a decreased LF/HF ratio, indicating increased vagal tone to

the heart. Moreover, in patients with severe heart failure, a 15-day course of daily tVNS almost doubled the 6-minute walk test distance [1]. This effect was coupled with a reduction in heart rate and improved NYHA functional class.

In addition to heart failure and CAD, tVNS has been investigated as a treatment of myocardial arrhythmias. Studies in anaesthetised dogs have demonstrated a protective effect of tVNS on atrial fibrillation (AF), following a sustained period of rapid atrial pacing [56, 394]. Furthermore, tVNS treatment in dogs that had recovered from a myocardial infarction significantly attenuated cardiac remodelling and improved left ventricular function. This effect has also been observed in the clinical setting; tVNS reduced the duration of AF in patients suffering from paroxysmal AF who presented for AF ablation [330].

The primary advantage for tVNS is its ease of application. Current clinical practise for vagus nerve stimulation involves a surgical procedure, under general anaesthetic, to insert a device around the left or right cervical vagus nerve. Studies investigating the merits of VNS in the cardiac setting have thus far proven ineffective [104, 399]. The surgery to insert the device carries significant risks, not least from the anaesthesia and proximity of the vagus nerve to the carotid artery. This poses an issue in particular for very sick or older patients. Moreover, the vagus nerve is known to carry a high number of sympathetic fibres. Indeed, when the cervical vagus nerve was stimulated in dogs, activity of neurones in the stellate ganglion increased [295]. This increased sympathetic nerve activity would clearly be detrimental to patients suffering from cardiovascular disease. Thus, current practise for VNS is likely too crude to produce an effective outcome. Perhaps tVNS could provide a viable alternative, given its specific recruitment of the parasympathetic nervous system and non-invasive application.



## **7.4 Aim 2: tVNS as a cardioprotective intervention in a rat *in vivo* model of myocardial infarction**

### **7.4.1 Background**

Given the data presented in section 7.3, we decided next to try and create a model of tVNS in the laboratory. This would allow more detailed investigation into the mechanism of action. The auricular branch of the vagus nerve appears to be well conserved in mammals; studies have demonstrated benefits of tVNS in cats, dogs, rats and mice [19, 155, 259, 330]. With respect to the rat, a tracing study revealed the ABVN is connected to parasympathetic regions of the brain [135], consistent with that seen in humans [96]. Moreover, in rat models of acute myocardial infarction, stimulation of the vagus nerve has been demonstrated to be highly cardioprotective [79, 174, 243]. These studies, however, all used invasive methods to access and stimulate the nerve. Two recent studies have investigated tVNS in a rat model of cerebral ischaemia-reperfusion injury [19, 165]. Electrical stimulation of the tragus or cymba concha decreased brain infarct size and improved post-infarction recovery, following 2hr middle cerebral artery occlusion. We therefore hypothesised that tVNS could ameliorate infarct size in a rat model of myocardial ischaemia-reperfusion injury.

### **7.4.2 Methods**

#### *In vivo model of myocardial ischaemia-reperfusion injury*

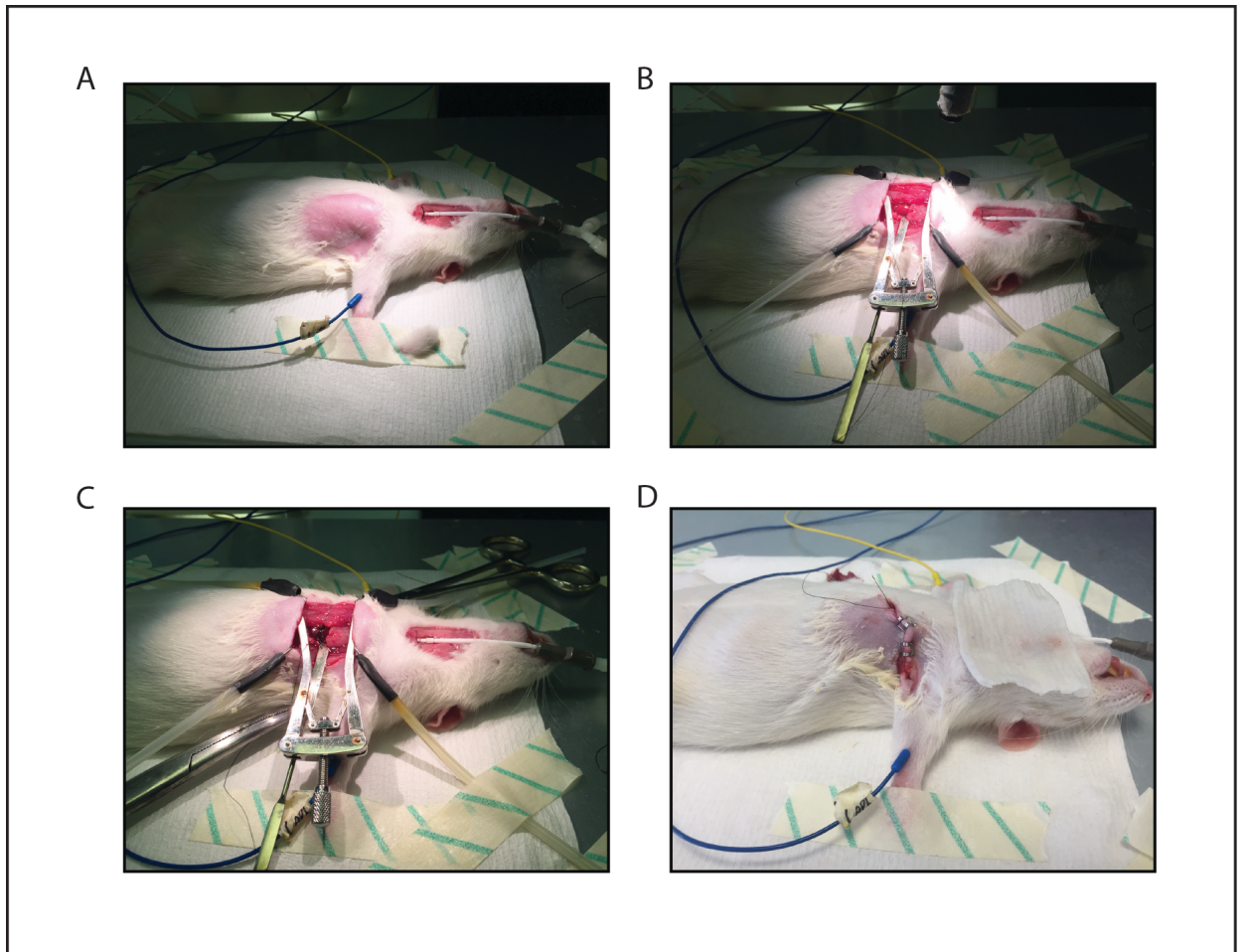
Male Sprague-Dawley rats were used throughout the study. Animals were anaesthetised with 60mg/kg sodium pentobarbital (i.p.) (Animalcare, York, UK). Surgery began once both pedal and tail reflexes were abolished. Depth of anaesthesia was monitored throughout the surgery, and additional pentobarbital was administered as required (25mg/kg, i.p.).

The animal was first arranged in a supine position on a temperature-controlled mat (Harvard Apparatus, Kent, UK), with tape securing all four limbs. A rectal temperature probe was inserted and body temperature

maintained at  $37\pm0.5^{\circ}\text{C}$ . A tracheostomy was performed and the trachea cannulated using a modified 16G, 1.7 x 51 mm Abbocath-T intravenous cannula (Smiths Medical International Ltd, Kent, UK). Positive pressure ventilation was provided by connection to a Small Animal Ventilator (Harvard Apparatus, York, UK). Tidal volumes were calculated using the following allometric formula: tidal volume = 7.2 ml/kg. Finally, 2 cmH<sub>2</sub>O of positive end expiratory pressure were added by submersion of the expiratory tubing in water. This is important to prevent lung collapse during open chest surgery.

Hair removal cream was applied to the left side of the thorax to facilitate surgery. A skin incision was made in the left lateral position, 1 cm below the forelimbs. Superficial muscles were sharp dissected. Blunt thoracotomy of the left fourth intercostal space was performed in order to gain access to the thoracic cavity. The pericardium was torn with splinter forceps (B. Braun, PA, USA). The LAD coronary artery was under-run with a 6-0 braided silk non-absorbable suture with 9.3 mm, 3/8 curved needle (Ethicon, C of Edin, UK) positioned approximately 2 mm below the tip of the left atrium. After passing loops of 3-0 braided silk (Ethicon, C of Edin, UK) over each arm of the suture to facilitate reperfusion, ischaemia was initiated by occlusion of the ligating suture.

Successful ischaemia was confirmed by the occurrence of ST-segment elevation in the ECG trace, myocardial blanching and hypokinesia of the anterior wall of the heart. Ischaemia, as indicated by ST-segment elevation, was maintained using surgical clamps as ballast on each end of the ligating suture. The open wound was covered by a moistened swap to prevent desiccation and heat loss. Following 35 min of ischaemia, reperfusion of the LAD territory was initiated by release of the occluding suture, which was left in place to facilitate subsequent analysis, and successful reperfusion confirmed by reversal of the ST-segment elevation and myocardial colour change. Following induction of reperfusion, the open wound was closed using metal surgical staples.

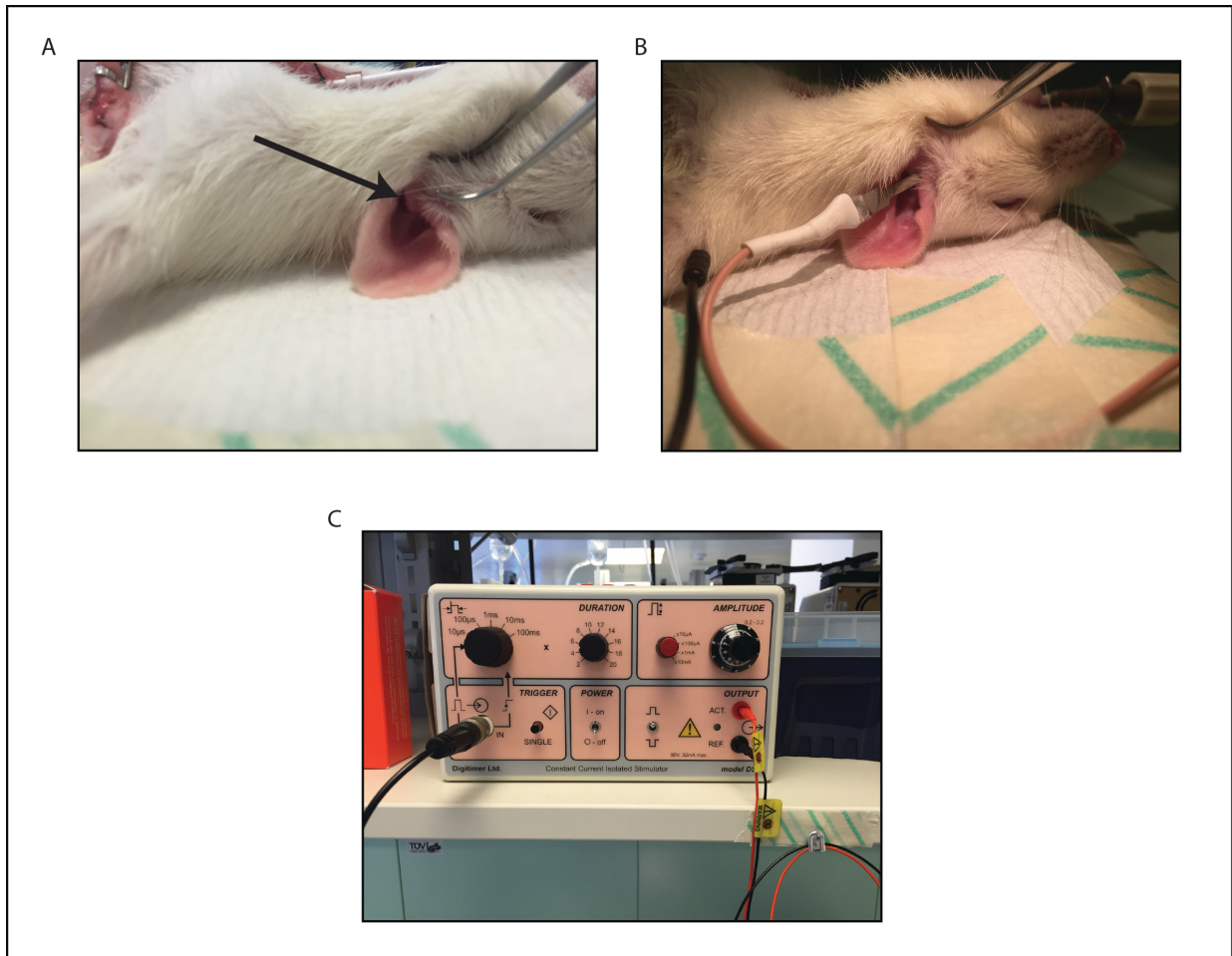


**Figure 7-6. Representative images of the surgical protocol for in vivo ischaemia-reperfusion injury model.** (A) The animal is anaesthetised, ventilated and arranged in a supine position on a temperature controlled mat; (B) The heart was access via thoracotomy of the intercostal space; (C) A suture was inserted around the left anterior descending coronary artery and tightened using a snare to occlude the artery and induce ischaemia; (D) Following 35min ischaemia, the suture was released to reperfuse the myocardium and the skin closed using surgical staples.

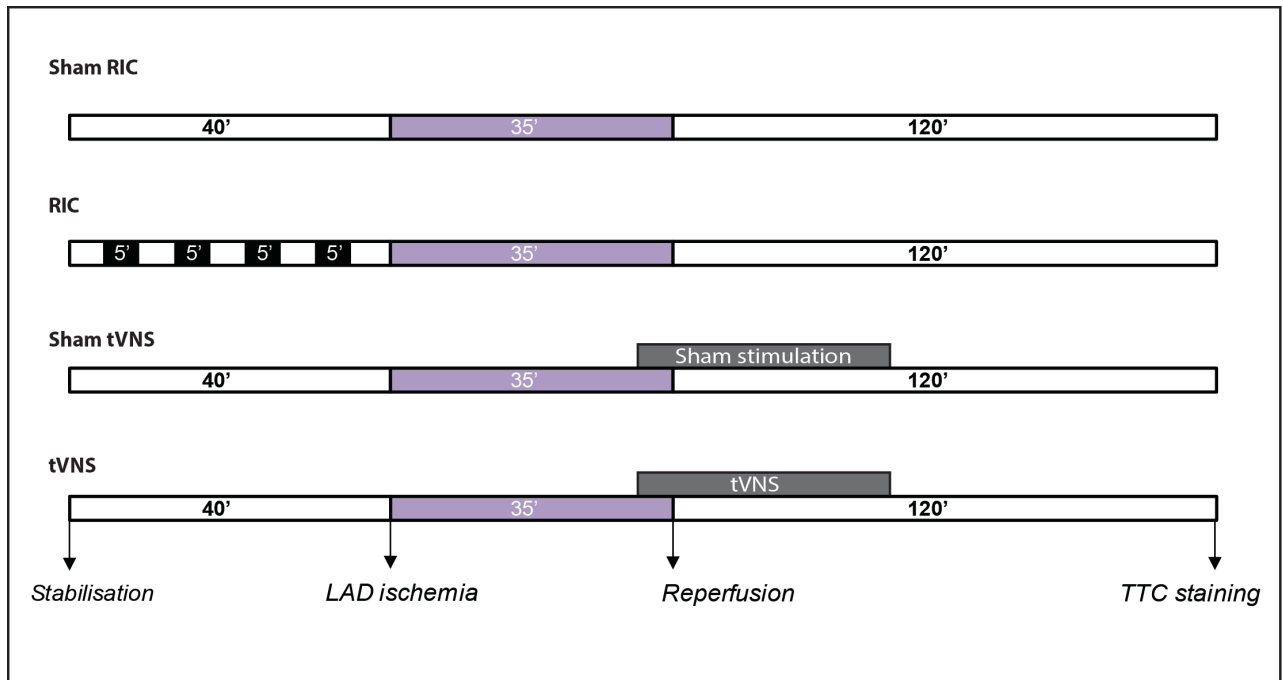
### *Experimental protocols*

A schematic of the experimental design is displayed in figure 7-8. All animals received 35min left anterior descending coronary artery ischaemia and 120min reperfusion. Prior to induction of ischaemia, animals were randomised to receive one of four separate interventions.

1. **Remote ischaemic conditioning (RIC):** induced via a modified pressure cuff placed around the left-hindlimb. This consisted of 4 x 5 min cuff inflation to 200 mmHg with intermittent 5 min deflations.
2. **Sham RIC:** where the cuff was applied to the animal for the same time period but not inflated.
3. **Transcutaneous vagus nerve stimulation (tVNS):** two modified stainless steel clips were attached to the left and right tragus of the animal (see Figure 7-7). These were connected to a constant current stimulator (DS3, Digitimer, Hertfordshire, UK). Stimulation was initiated 5min prior to reperfusion; a 30sec train consisting of 0.5ms pulse duration at 0.5mA and 20Hz. Stimulation was applied every 5min for 1 hour. This protocol was taken from that used in a recent study that demonstrated the neuroprotection induced by tVNS [19].
4. **Sham tVNS:** where the clips were attached to the left and right tragus without any electrical stimulation.



**Figure 7-7. Method used for transcutaneous vagus nerve stimulation in the anaesthetised rat. (A)** The rat ear has a clear tragus (indicated with an arrow); **(B)** A modified metal clip could be attached to the tragus; **(C)** Current was driven through the clip by a Digitimer DS3 constant current stimulator.



**Figure 7-8. Experimental protocols used to investigate RIC and tVNS in a rat model of ischaemia-reperfusion injury.** All rats received 35min left anterior descending coronary artery ischaemia and 60min reperfusion. Remote ischaemic conditioning (RIC) consisted of 4 cycles of 5min hindlimb ischaemia, induced via a small cuff, with intermittent 5min reperfusion. Transcutaneous vagus nerve stimulation (tVNS) consisted of two small clips attached to the left and right tragus of the rat; stimulation was started 5min prior to reperfusion and continued for 1 hour.

### *Infarct analysis*

Following reperfusion, a clamshell thoracotomy was performed and the heart excised as described in section 2.2.2. The aorta was cannulated and perfused with 5mls cold phosphate-buffered saline solution to clear the heart of blood. Infarct size was then assessed using the method described in section 2.2.5.

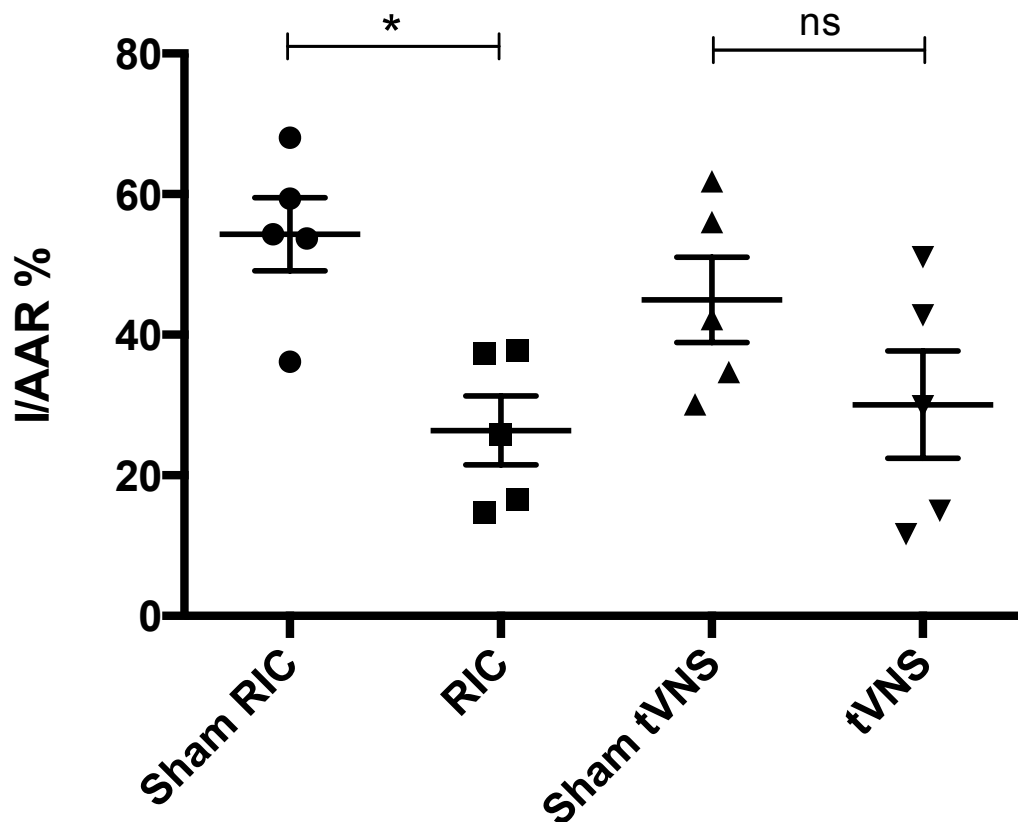
### *Statistics*

Data are presented as mean  $\pm$  standard error, and were analysed using a one-way analysis of variance. A p value of less than 0.05 was considered significant.

### 7.4.3 Results

#### *In vivo tVNS does not appear to significantly reduce infarct size*

Figure 7-17 displays the infarct sizes across all four experimental groups. Those animals that received RIC were significantly protected from ischaemia-reperfusion injury relative to sham RIC (I/AAR% =  $26 \pm 5$  vs Sham RIC =  $54 \pm 5$ ,  $p > 0.05$ ). Those animals who received tVNS had a mean infarct size of  $30 \pm 8$  %. Although this was lower than those who received sham tVNS ( $45 \pm 6$  %), it was not found to be statistically different ( $p = 0.17$ ).



**Figure 7-9. Remote ischaemic preconditioning but not tVNS reduces infarct size in an animal model of in vivo ischaemia-reperfusion injury.** The chart displays infarct size, expressed as a proportion of area at risk (I/AAR%), for each experimental group. All data presented as mean $\pm$ SEM. Those animals that received remote ischaemic conditioning (RIC) were significantly protected from ischaemia-reperfusion injury. Animals that received transcutaneous vagus nerve stimulation (tVNS) displayed a reduced infarct size relative to sham tVNS, however this was not statistically significant.

#### 7.4.4 Discussion

This study failed to demonstrate a cardioprotective effect of tVNS in a rat model of myocardial ischaemia-reperfusion injury. Although a reduction in infarct size was observed in animals that received tVNS, it was not statistically significant. RIC induced significant cardioprotection, consistent with the literature, thus acting as a positive control for the model.

The tVNS protocol used in this study was chosen to match that used in a recent neuro-protection study by Ay and colleagues [19]. Electrical stimulation of the tragus reduced infarct size in a rat model of cerebral ischaemia-reperfusion injury. This is the only study in the literature that has demonstrated a measurable effect of tVNS in a rat model. Moreover, the stimulation was given as a per-/post-conditioning intervention, as this more closely aligns with its potential clinical application.

This result is at odds with the human data presented in the previous section, where tVNS induced release of a cardioprotective blood-borne factor. Despite the obvious difference in species, there are two other key issues that could explain the different outcomes. First, the stimulation parameters used in the animal study are different to those used in the human study. As described above, this decision was made to align the rat study more closely to the published study that also used a rat model. The amplitude and frequency of stimulation was significantly different, and this could explain the lack of protection observed in the rat. Secondly, it is possible that the dialysate model does not provide adequate information to suggest a cardioprotective effect. That is, release of a cardioprotective blood-borne factor might not be sufficient to reach threshold for cardioprotection *in vivo*.

These data should therefore be considered only preliminary for several reasons. First, there was a large amount of variability in infarct size with rats following tVNS. Indeed, one could argue that protection did not occur in two of the five animals (Fig. 7-9). The key question, therefore, is whether adequate stimulation was achieved in all five animals. The tragus is relatively small in the rat (Fig. 7-7), therefore it is difficult to ensure good conduction



between the stimulating clip and tragus. There are currently no biomarkers that indicate appropriate tVNS stimulation. Thus, it is highly possible that the strength of tVNS varied between each animal. Moreover, only one stimulation protocol was used in the study. With IPC and RIC, a threshold must be reached in order for cardioprotection to occur; there exists an optimal protocol for both interventions in order to achieve the maximum effect [46, 368]. Given the reduction in infarct size observed with tVNS, albeit non-significant, it is important to test different stimulation parameters before drawing firm conclusions as to its cardioprotective potential. Perhaps, there exists a protocol that sufficiently activates the vagus nerve so as to reach the threshold for cardioprotection.

One solution could be to measure cervical vagus nerve activity during tVNS in the anaesthetised rat. If the tVNS is working via increased vagal tone, this would be detected as increased cervical vagus activity. The rat could then be infarcted, and the infarct size correlated against vagus nerve activity following tVNS; the hypothesis being that higher vagus nerve activity following tVNS would be associated with lower infarct size.

## **7.5 Summary**

This chapter has introduced transcutaneous vagus nerve stimulation as a potential novel cardioprotective intervention. Dialysate prepared following tVNS in human volunteers significantly protected a naïve-isolated rat heart from ischaemia-reperfusion injury. In addition, remote ischaemic conditioning produced a dialysate that protected a naïve heart to a similar level. This suggests that tVNS activates a similar mechanistic pathway to RIC, both inducing release of a blood-borne cardioprotective factor. In the subsequent study, tVNS did not reduce infarct size in an in vivo model of rat ischaemia-reperfusion injury. A small reduction in injury was observed, however, which warrants further investigation into the optimal stimulation parameters.

It is very important to develop an animal model for tVNS for two reasons: first, it allows detailed investigation into the mechanism of action; second, one can easily investigate and determine the optimal tVNS protocol for

cardioprotection. Our data do not allow a firm conclusion as to the potential for tVNS as a cardioprotective intervention, but provide an interesting starting point requiring further investigation.

## **Chapter 8 General conclusions**

The overall aim of this thesis was to investigate the mechanisms by which remote and classical ischaemic conditioning communicate their protective message. Particular focus was given to a neuro-humoral pathway, with key similarities observed between the mechanisms of both IPC and RIC. Indeed, preliminary data presented in this thesis suggest an important role for the intrinsic cardiac nervous system in response to blood-borne factors released following both interventions. Moreover, the vagus nerve was found to be a fundamental communicative step between the induction of RIC in the limb and release of a humoral mediator. We suggest that there exists a complex interaction between the sensory perception of brief ischaemia-reperfusion injury and release of cytoprotective humoral factors into the blood.

A large portion of time during this PhD was spent developing and characterising the two models of cardioprotection; the IPC effluent transfer model and RIC plasma dialysate model. In doing so, this not only created the platform to investigate our hypotheses, but also enabled a thorough understanding of the nature of the two cardioprotective interventions. The effluent model demonstrated that humoral factors were released following IPC that induced vasodilation and cardioprotection to a naïve heart. The dialysate model revealed a humoral component to RIC and enabled investigation into the mechanism of its release and induction of protection in the myocardium.

Using the dialysate model, we revealed that bilateral cervical vagotomy abrogated release of the humoral blood-borne mediator (Chapter 5), which added to the current neuro-humoral paradigm for the communication of RIC from the conditioned limb to the myocardium. This result is complimented

nicely with two other recent studies; the suggestion is that RIC triggers increased vagal tone to the gut, releasing glucagon-like peptide 1 into the blood, which then moves to the heart and induces protection. However, dialysate prepared following stimulation of the gastric vagal nerve did not protect a naïve heart (section 5.3.2.2), indicating release of a factor did not occur. Thus, this hypothesis remains equivocal and requires further experimentation. We further demonstrated that the humoral RIC mediator appears to recruit intrinsic cardiac ganglia, in as much as protection was abrogated when hexamethonium was co-infused with the plasma dialysate.

These data prompted the question of whether intrinsic cardiac ganglia were important in the mechanism of classical ischaemic preconditioning (IPC). Indeed, pretreatment with the ganglionic antagonist hexamethonium abrogated the cardioprotection of IPC in the isolated perfused heart. Moreover, we measured a release of acetylcholine in coronary effluent following IPC, and the muscarinic antagonist atropine also abrogated IPC. This indicated that intrinsic neural pathways remain active in the isolated heart preparation and can influence cardioprotection in the absence of any input from the central nervous system.

Perhaps the most surprising result was presented in Chapter 7, where stimulation of the tragus in the ear induced release of a blood-borne cardioprotective factor. However, we were not able to reproduce these data in an *in vivo* rat model of myocardial infarction. Thus, this chapter should be considered preliminary data, however tvNS represents a highly interesting intervention that merits further pre-clinical investigation.

As a final point, the meta-analysis (Chapter 4) highlighted one of the key pitfalls of translational medicine; the rush to perform a clinical trial in the absence of sufficient pre-clinical data. We were only able to find 31 studies investigating RIC in animal *in vivo* models of myocardial infarction. Given the intervention was discovered in 1993, this rate of just over one study per year is insufficient when considering the number of clinical trials using RIC in the same period. Indeed, there remains a lack of clarity as to the most effective

protocol for RIC. In addition, the recent discovery of a key role for the vagus nerve in RIC could explain the apparent lack of efficacy in the clinic; vagal tone is known to depreciate in patients suffering from coronary artery disease (CAD). However, despite recent setback in large clinical outcome studies, RIC remains a clinically feasible, non-invasive, inexpensive intervention with the potential to improve outcome in CAD patients.

## Chapter 9      References

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## **Chapter 10      Appendix**

Below are attached the three original research publications arising from data presented in this thesis. Note that the page numbers and references correspond to the individual manuscripts, not to the overall document.